REMARKS

Claims 1 and 3-7 are pending. No new matter has been added by way of the present amendments. For instance, claims 1 and 7 have been amended to specifically define the ginsenoside glycoside as being at least one selected from ginsenoside Rg_3, Rg_5 and Rh_2 as supported by originally filed claim 2 as well as the present specification at page 9, second full paragraph. Claims 1 and 7 have also been amended to define the type of cancer as lung cancer as supported by the present specification at, for example, pages 1-3, in particular Tables 1 and 2, as well as Table 4 on page 6, and page 7, lines 10-24. The scope of claim 3 was clarified and the dependency of claims 4, 5 and 6 was altered. Claims 2 and 8-10 were cancelled. Also, the specification was amended to properly cross-reference the parent application. Although a similar cross-referencing amendment was requested in the Transmittal letter of the present Divisional, Applicants wish to make certain that the amendment is entered on the record. Accordingly, no new matter has been added.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all rejections and allow the currently pending claims.

Issues under 35 U.S.C. §112, first paragraph

The Examiner has rejected claims 1-6, 9 and 10 under 35 U.S.C. §112, first paragraph for the reasons recited at pages 2-4 of the outstanding Office Action. Applicants respectfully traverse this rejection.

The Examiner has asserted that undue experimentation would be required to practice the present invention as claimed. Applicants disagree.

Independent claim 1 relates to a method of prophylactically treating lung cancer comprising administering to a subject in need thereof a pharmaceutically acceptable amount of a composition that comprises at least one ginsenoside glycoside selected from the group consisting of ginsenoside Rg_3, Rg_5, and Rh_2 as an active ingredient(s) and a pharmaceutically acceptable carrier or adjuvant. Independent claim 7 relates to method of decreasing the occurrence in the incidence of lung cancer comprising the same step as claim 1.

The Examiner has asserted that the claiming of "prevention" of cancer is non-enabled. The Examiner has also alleged that the Experiments in the specification are insufficient to show cancer prevention using ginsenosides. Applicants disagree with the Examiner and offer the following analysis for consideration. A review of the following remarks, as well as the present specification will reveal that those of skill in the art are fully

enabled to make and use the presently claimed invention without undue experimentation.

Applicants first address the use of the word "prevent" as cited by the Examiner at page 3, lines 8-10 of the outstanding Office Action. The Examiner has asserted that a simple dictionary definition of "prevent" shows that this word means that a situation must be kept from occurring (see Merriam-Webster's Collegiate Dictionary (1997), 10th ed. Page 924). Apparently, the Examiner believes that the word "prevent" according to Webster's Collegiate Dictionary (1997), 10th ed. Page 924 is decrypted as "keep from occurrence". Further, allegedly to prevent occurrence of cancer to some extent is not regarded as proper example of "prevention."

However, Applicants point out that in the Medical Dictionary used by medical doctors, who are those of skill in the art, (refer to Dorland's Illustrated Medical Dictionary (2000), 29th ed. Page 1455), "preventive" is defined as "serving to avert the occurrence of" and "to prevent with chemicals" is defined as "chemoprevention" or "chemoprophylaxis". In the cited dictionary "chemoprophylaxis" is defined as "use of a chemotherapeutic agent as a means of preventing development of a specific disease." A copy of these definitions is attached.

In addition, Dr. Gary J. Kelloff (Chief of Division of Cancer Prevention, Chemoprevention Brach, National Cancer Institute) defined in *Ann. New York Acad. Sci.*, vol. 889, pp 1, 1999 (copy

attached) that "Cancer chemoprevention is defined as the use of specific chemical compounds to prevent, inhibit, or reverse carcinogenesis." Therefore, Applicants submit that the term "prevent" is not always interpreted as to "keep from occurrence" but rather also "inhibition of carcinogenesis" is regarded as "cancer prevention."

In addition, humans are not exposed even under 1/10,000 of the concentration used in the present experiment and it is aim that researcher of cancer chemoprevention prevent even 1% of occurrence of human tumors.

Applicants now address the Examiner's assertion that Applicant has allegedly not shown that isolated ginsenosides are able to keep cancer from occurring. The Examiner has asserted that Applicants' specification has one experiment with rats that attempts to show the cancer preventive effects of ginsenosides (Experiment 1). The Examiner admits that this experiment shows a reduction in the amount of cancers, but a significant number of rats given the carcinogen in combination with the ginsenoside still contract cancer. Thus, the Examiner alleges that Applicants' own specification shows that the ginsenosides do not prevent cancer. Applicants respectfully disagree with the Examiner's analysis of the present specification.

Applicants have used a mouse model, not a rat model as alleged by the Examiner. In particular, Applicants have injected

benzo(a)pyrene subcutaneously 500μg per new born mice within 24 hours after birth. The dose of benzopyrene would be more than 12 times of usual dose of 40ug to have early occurrence of lung tumors in 9 week after birth instead of 40 weeks.

All mice were sacrificed at the 9th week after birth. Lungs were excised and fixed in fixing solution and the tumors were counted. To obtain an index of tumor incidence, the percentage of tumor bearing mice per total number of mice in each group was calculated. Statistical comparisons were made using the Chi-square test for tumor incidence. A null hypothesis was rejected whenever a P value of 0.05 or less was found (see Yun et al., *Anticancer Pre.*, 15: 839-846, 1955, attached).

This experimental method is a method designed such that in the control group dosed with chemical carcinogen, lung tumors occurred in about 50% of the mice. Therefore, 500μg of chemical carcinogen, that is, 12 times of common dosage is dosed and resulted in a 50% occurrence of lung tumor in a short time (6 weeks). After weaning of mice after 3 weeks of birth, ginsenosides of the present invention are dosed for 6 weeks and resulted in a 20 - 40% suppression of lung tumors. It is judged that a strong preventing action is statistically exhibited when meaningful P value < 0.05 is obtained.

Accordingly, even when 12 times of carcinogen of common dosage is administered, lung tumors do not occur at 100% of the mice. On

the other hand, compared with lung tumors occurring in 50% of mice dosed with a large volume of carcinogen, the fact that the group in which ginsenosides are dosed in combination with the carcinogen, occurrence of lung tumors is decreased 20-40%. Applicants submit that this is a very strong inhibition, when considering the volume of carcinogen. Further, from the result of the experiment, a decrease of 20% is statistically meaningful.

To summarize, the Examiner's attention is focused on the occurrence of lung tumors by administering a large volume of carcinogen and the suppression thereof by dosing of ginsenosides. This remains a meaningful results regardless of the lack of suppression of lung tumors in many of the mice by dosing ginsenosides in combination. Those of skill in the art understand these results to be meaningful and would have no undue experimentation in the implementation thereof.

The Examiner has also asserted that Applicants' experiment only shows the reduction of specific types of cancer using rats as model, while the claims are drawn to reducing any type of cancer in any patient. This is asserted to encompass treating humans.

Applicants have observed that in the humans dosed with a heat-treated red ginseng or a water extract of ginseng, a remarkable decrease of cancer risk resulted. This included two experiments of 905 pairs case-control, 1987 pairs case-control study and cohort study at ginseng cultivation area. This was all

prior to carrying out the present ginsenoside experiment 1. Accordingly, experiment 1 of the present invention was carried out with ginsenosides existing in red ginseng.

Applicants surmised that suppression of the occurrence of lung cancer by ginsenosides Rg_3, Rg_5 and Rh_2 is obtained by the effect of ginsenoside Rg_3, Rg_5 and Rh_2 contained in red ginseng. Thus, Applicants judged that as considered from the above epidemiological results, ginsenoside Rg_3, Rg_5 and Rh_2 are effective on prevention of all cancers or tumors. However, the Examiner's attention is drawn to the fact that the present claims are drawn to the treatment of lung cancer in particular.

The Examiner has also pointed out at page 4 of the outstanding Office action that Prior art such as Gorman and Golden (Time Magazine (1998), vol. 151, no.19, pp.38-44) and Gura (Science (1997), vol. 278, pp.1041 and 1042) show that it is known in the art that rodent models are not considered to be predictive of human treatment. The Examiner has asserted that at page 44, Golden discusses that research scientists in the cancer community acknowledge that "most drugs that work in lab animal turn out to be duds in human." The Examiner also points out that Gura quotes cancer drug researchers as stating "that the fundamental problem in drug discovery for cancer is that the model systems are not predictive at all (see page 1041, first column, second paragraph)."

Based upon these references, the Examiner asserts that there exists unpredictability in applying rodent results to human treatment, therefore, a person of ordinary skill in the art would allegedly be forced to experiment unduly in order to ascertain if the claimed invention can function as claimed in any patient other than in the model used. Applicants disagree.

In 1983, investigators established a 9-week, medium-term anti-carcinogenicity model based on the incidence of mouse pulmonary adenomas induced by benzo(a)pyrene, termed Yun's model. Ascorbic acid, beta-carotene, and red ginseng extract were tested. Surprisingly, there was no anti-carcinogenic effect of beta-carotene, but ascorbic acid and red ginseng extract had positive effects. This result was withheld as unexplainable for 5 years and then published (T.K. Yun et. Al. J. Korean Cancer Assoc., 19: 1-7, 1987), when a preliminary report of the Physician's Health Study in the USA showed negative results with beta-carotene (The Steering Committee: N. Eng. J. Med., 318: 262-264, 1988).

These results were consistent with the lack of efficacy observed in a trial of 29133 randomly selected male smokers (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group: N. Engl. J. Med., 330: 1029-1035, 1994), a study of more than 18,000 people at high risk of lung cancer (Omenn, G.S. et. al.: J. Natl. Cancer Inst., 88: 1550-1559, 1996; N. Engl. J. Med., 334: 1150-

1155, 1996), and the Physicians' Health Study of 22 071 American physicians(Henekens, C.H.: N.Engl. J. Med., 334: 1145-1149,1996).

However, at the recommendation of the Chemoprevention Branch, Division of Cancer Chemoprevention and Control, at the U.S. National Cancer Institute, Korean researchers tested the effect of red ginseng extract on models with azoxymethane-induced colon cancer and N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced bladder cancer. The results were negative (Unpublished). Moreover, 13-cis retinoic acid was also without benefit in the 9-week medium-term study (Yun, T.K. et. al.: Anticancer Res., 15: 839-846, 1995).

The 9 week medium-term anti-carcinogenicity test model in mouse lung cancer contributed greatly to the testing of ginseng's effects. The results obtained have repeatedly been confirmed by other methods. However, because rectal cancer induced by azoxymethane and bladder cancer induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine were not inhibited by ginseng (unpublished), the investigators who use these two models may not believe the effects of ginseng.

Recently, alteration of genes has been found in mouse lung tumors, as in human lung cancer, and the mouse tumor has therefore been suggested as a preclinical model of chemopreventive agents for human lung cancer. Dr. Herzog's article is of particular interest (see Herzog, C.R. at al., Genetic alterations in mouse lung tumors:

Implications for cancer chemoprevention, J. Cell Biochem. Suppl. 28/29: 49-63, 1997), copy attached.

Specific genetic alterations affecting known tumor suppressor genes and proto-oncogenes occur during mouse lung tumorgenesis. These include mutational activation of the K-ras gene, commonly seen at a frequency of about 80% in both spontaneously occurring and chemically induced adenomas and adenocarcinomas of the lung, suggesting that it an early event that persists into malignancy. allelic loss of the p16 tumor in about 50% of mouse lung adenocarcinomas, but rarely in lung adenomas, suggesting that it may play a role in malignant conversion or progression of lung tumors.

The mouse lung tumor model has become a valuable alternative or identifying such genes. Recently, loci responsible for mouse lung tumor susceptibility have been mapped to chromosome 6, 9, 17 and 19, while those linked to lung tumor resistance have been mapped to chromosomes 4, 11, 12, and 18. Known candidate susceptibility or resistance genes include the K-ras overlap between the genetic alterations that underlie human and mouse lung tumorgenesis, the mouse lung tumor model has been expanded to include pre-clinical screening of chemopreventive agents against human lung cancer. Studies on the modulation of genetic defects in mouse lung tumors by known and potential chemopreventive agents

should further the goal of developing an effective prevention and treatment of lung cancer.

Dr. You's article is also relevant (see You, M. et al., Preclinical and clinical models of lung cancer chemoprevention, Hematol. Oncol. Clin. North Am., 12: 1037-1053, 1998), copy attached. Because the histopathological changes, stages of tumor progression, and molecular changes in mouse lung adenocarcinomas are similar to those in human lung adenocarcinomas, the mouse lung tumor model has been used extensively to evaluate the efficacy of putative lung cancer chemopreventive agents.

The selection of beta-carotene as a potential chemopreventive for the lung was based largely upon epidemiological data that showed a positive correlation between the consumption of beta-carotene-rich foods, and high blood levels of beta-carotene, retinoids or vitamin E were initiated subsequently, but none of them showed any chemopreventive effects. The complex relationship between chemoprevention and smoking was illustrated in the Alpha-Tocopherol, Beta-carotene Cancer Prevention Study (ATB) completed in Finland and in the Beta-carotene and Retinol Efficacy Trial (Caret) in the United States. The results showed that these supplements alone or in combination with retinal increased lung cancer risks in smokers.

The Physicians' Health Study involved about 22,000 American physicians who were randomized into two populations. The first

received 50mg of beta-carotene on alternative days, and the second group received a placebo. During the 12-year study, the difference that was not significant.

Other chemicals that were ineffective against mouse lung tumor development include 9-cis-retinoic acid, 4-HPR, and oltipraz (Lubet et al, unpublished data).

Dr. You has summarized the above findings as follows:

In smokers, beta-carotene, retinoid, and vitamins E and C appear to have little or negative effect against human lung cancer development. Similarly, these chemicals have generally failed to inhibit lung tumorgenesis in mice. Recently, alteration of genes has been found in mouse lung tumors, as in human lung cancer, and the mouse tumor has therefore been suggested as a preclinical model of chemopreventive agents for human lung cancer (Herzog, C.R. et al.: J. Cell Biochem. Suppl. 28/29: 49-63, 1997; You, M. et al.: Hematol. Oncol. Clin. North Am., 12: 1037-1053, 1998).

According to Dr. You, although epidemiological evidence exists that consumption of fruits and vegetables containing compounds such as beta-carotene may reduce lung cancer risk, beta-carotene in combination with retinol did not protect lung cancers from carcinogen induced carcinogenesis in mice.

These results are consistent with several clinical trials conducted later using the same combination of agents in humans, in which an increased incidence in lung cancer was observed in

smokers. Other chemicals that were ineffective against mouse lung tumor development include 9-cis-retinoic acid, 4-HPR, and oltipraz (Lubet et al, unpublished data).

In summary, Applicants respectfully submit that the mouse model used in the present application is reliable and thus, the present claims are fully enabled. Reconsideration and withdrawal of the outstanding rejection are respectfully requested.

Issues under 35 U.S.C. §102(b)

The Examiner has rejected claims 7 and 8 under 35 U.S.C. §102(b) as being anticipated by Yun et al., *Cancer Epidemiology Biomarkers & Prev.*, vol. 4, pp. 401-408, (1995). Applicants respectfully traverse.

Applicants respectfully submit that the Yun reference is a thesis concerning the fact that in the humans dosed with heat-treated red ginseng, raw ginseng (raw ginseng, ginseng juice or extract of raw ginseng with hot water), dried ginseng (powder or extract of dried ginseng with hot water), there was reported a decrease in cancer risk in two experiments of 905 pairs case-control, 1987 pairs case-control study and cohort study at ginseng cultivation area. However, the Yun reference fails to suggest or disclose the use of ginsenosides Rg_3, Rh_2 and Rg_5 as required by independent claim 7. Accordingly, there exists no anticipation

based upon Yun. Reconsideration and withdrawal of this rejection are respectfully requested.

In view of the above, Applicants respectfully submit that the currently pending claims are in condition for allowance. The Examiner is therefore requested to withdraw all outstanding rejections and allow the currently pending claims.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Craig A. McRobbie (Reg. No. 42,874) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition for a two (2) month extension of time for filing a reply in connection with the present application, and the required fee of $225.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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0662-0189P

Attachments:

(1) Curriculum vitae of Taik-Koo Yun, M.D., Ph. D., one of the inventors and is a cancer researcher who has more than 45 years of experience in cancer research.

(2) Excerpts from Dorland's Medical Dictionary (2000).

(3) Dr. Gary J. Kelloff (Chief of Division of Cancer Prevention, Chemoprevention Brach, National Cancer Institute) defined in *Ann. New York Acad. Sci.*, vol. 889, pp 1, 1999.

(4) Herzog, C.R. at al., Genetic alterations in mouse lung tumors: Implications for cancer chemoprevention, J. Cell Biochem. Suppl. 28/29: 49-63, 1997.

(5) You, M. et al., Preclinical and clinical models of lung cancer chemoprevention, Hematol. Oncol. Clin. North Am., 12: 1037-1053, 1998.

CURRICULUM VITAE

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Academic Training

1945. 04. 01.- 1951. 08. 31 : Kyunggi Middle and High School, Seoul
1951. 05. 01.- 1953. 03. 27 : Premedical Course, College of Liberal Arts, Seoul National University (SNU).
1953. 04. 01.- 1957. 03. 28 : College of Medicine, SNU
1957. 04. 01.- 1959. 03. 29 : Master Course in Pathology, Medical Science, Postgraduate School, SNU
1959. 04. 01.- 1964. 02. 26 : Ph. D Course in Postgraduate School, SNU

Research & Teaching Experiences

1957. 06. 15. : Appointed as Army Surgeon Leutenant
1957. 10. 01. - 1959. 10. 11 : Comissioned as the army surgeon and dispatched to the Department of Pathology, College of Medicine, SNU.
1959. 10. 12. - 1962. 03. 30 : Pathologist, Laboratory Services, Capital Army Hospital, Seoul
1962. 03. 01. - 1964. 07. 31 : Chief, Laboratory Services, Capital Army Hospital, Seoul (Major).
1964. 12. 01. : Specialty Board in Anatomical Pathology and Clinical Pathology
1964. 12. 01. - 1968. 03. 10.: Researcher(3 Kap), Department of Radioisotopes, Radiological Institute, Office of Atomic Energy, Seoul
1968. 03. 01. - 1969. 09. 25.: Chief(2 Eul), Laboratory of Pathology, Radiological Research Institute, Office of Atomic Energy, Seoul.
1968. 04. 01. - 1969. 09. 25.: Visiting Assistant Professor, Department of Pathology, College of Medicine, SNU. :
1969. 10. 01. - 1971. 06. 31.: U.S. Public Health Service Fogarty International Postdoctoral Research Fellow, Laboratory of Pathology, National Cancer Institute(NCI), National Institutes of Health, Bethesda, MD, U.S.A.

1971. 07. 01. - 1973. 02. 16.: Returned to Chief(2 Kap), Laboratory of Pathology, Radiological Research Institute, Office of Atomic Energy, Seoul.

1973. 02. 17. - 1994. 01. 14. : Chief, Laboratory of Cancer Pathology, Cancer Research Hospital, Re-organized Korea Atomic Energy Research Institute(KAERI), Seoul.

1974. 03. 20. - 1978. 02. 28. : Visiting Associate Professor, Department of Pathology, College of Medicine, SNU.

1975. 08. 30. - 1985. 07. 30. : Visiting Professor, Department of Pathology, College of Medicine, Chungang University, Seoul.

1978. 03. 01. - 1992. 08. 31. : Visiting Professor, Department of Pathology, College of Medicine, SNU.

1979. 01. 18. - 1988. 03. 28. : Director, Division of Research Developments, Cancer Research Hospital, KAERI, Seoul.

1980. 08. 25. - 1989. 12. 31. : President, Korea Cancer Center Hospital(KCCH), KAERI, Seoul.

1994. 01. 14. - 1994. 12. 31.: Principal Investigator, Laboratory of Cancer Pathology, KCCH, KAERI, Seoul.

1995. 01. 01. - 1997. 12. 31 : Principal Investigator, Laboratory of Experimental Pathology, KCCH, KAERI, Seoul.

1997. 12. 31. : Retired from Korean Cancer Center Hospital, Seoul.

1998. 01. 01. - 1999. 04. 31.: Invited Researcher, Laboratory of Experimental Pathology, Korea Cancer Center Hospital, KAERI, Seoul.

2002. 05. 01. - Present : President, Korea institute of Cancer Chemoprevention, Seoul.

Professional Experiences and Society Activities

1977. 01. 29. - 1980. 12. 31. : First and Second President, Korean Society of Environmental Mutagen and Carcinogen

1982. 03. 30. - 1987. 06. 03.: Committee, Planning of Safty Control Branch Committee, Committee of Safty Policy Development on Drug Efficacy and Pharmaceuticals, Central Committee of Drug Evaluation in Ministry of Public Health, Korea.

1982. 12. 01. - 1986. 01. 30. : Committee, Central Committee of Environmental Preservation, Ministry of Environmental Affairs.

1983. 02. 01. -1989. 04. 09. : Chairman, Board of Directors, Korean Cancer Society

1983. 02. 01. – 1994. 09. 15. : Board of Directors, Korean Society of Ginseng Research

1983- 03. 01. - Present : Board of Directors, Society of Alma Mater, College of Medicine, SNU.

1985. 05. 01. - Present : Board of Directors, Society of Alma Mater, Graduate School of SNU.

1989. 07. 01. - 1995. 04. 24. : Vice President, Korean Cancer Society

1991. 11. 17. - 1991. 11. 08. : President, Korean Society of Immunology

1993. 06. 11. - 1994. 06. 10. : Vice President, Korean Cancer Association

1994. 06. 10. - 1995. 06. 09. : President, Korean Cancer Association
1994. 11. 22. - Present : Inaugural Active Member, Korean Academy of Science and Technology
1995. 04. 25. - 1997. 06. 15. : President, Korean Cancer Society
1995. 05. 12. - 2000. 12. 10 : Vice President, Korean Society of Ginseng Research
1995. 05. 13. - 1997. 12. 30. : Committee, Committee of 10 Year Planning on Cancer Conquering, Ministry of Public Health, Korea.
1995 - Present : Fellow , Division of Medical Sciences, Korean Academy of Science and Technology.
1996. 07. 13. - 2000. 11. 17. : First and Second President, Korean Association of Cancer Prevention
1996, 10. 15. - Present : Active member, Amercian Association of Cancer Research(AACR).
1997. 04. 05. - Present : Inaugural Active Member, Korean Association of Advancement of Sciences, Seoul.
1997. 04. 05. - 1999. 12. 30.: Active member, New York Academy of Sciences
1999.03. 29. - Present :Board Member, International Society of Cancer Chemoprevention (ISCaC), New York, U.S.A.
2000.11. 25. – 2001. 07. 30. : Chairman, Organizsing Committee, International Symposium on Cancer Chemoprevention of INSAM(Ginseng), Seoul, Korea, supported by Korean Association of Medical Sciences.
2000. 12. 17. – 2002. 11. 19 : President Emeritus, Korean Association of Cancer Prevention

Honors and Awards

1968. 04. 21. : Awarded on Contracted Research of Ministry of Public Health on 1st Science Day
1969. 10. 01. : Awarded the U.S. PHS International Fogarty Postdoctoral Fellowship
1973. 10. 05. : Awarded the Academic Honor in Basic Medical Science by Korean Medical Association on 21st Annual Meeting of Korean Medical Association.
1973. 10. 18. : Awarded on Exhibition of Inbred and non-inbred experimental animals, at 1st Science Exhibition held by Koream Medical Association.
1976. 04. 21. : Conferred a National Medal(Cammellia Grade) by the President of Republic of Korea in 9th Science Day.
1986. 03. 01. : Awarded the 3.1 Cultural Medal for Academic Honor in Medical Science on 72nd 3.1 Movement Anniversary Day by 3.1 Cultural Foundation.
1993. 09. 17. : Awarded the National Academy of Sciences, Korea. (Applied Natural Sciences Areas)
1997.04. 13 : Winner of the Ochi Award for Functional Food Research, Symposium on Functional Foods Held by 213th National Meetings of the American Chemical Society(ACS), San Francisco, Ca. USA.
1998.12. 11 : Awarded the Academic Honor by Korean Society of Ginseng Research.

Listed in Who's Who

1987 : Listed in "International Who's who of Intellectuals" Seventh Edition, published by International Biographical Centre, Cambridge, England.

1990 - Listed in "*Who's Who in America*" published by Marquis.

29th
EDITION

DORLAND'S Illustrated MEDICAL DICTIONARY

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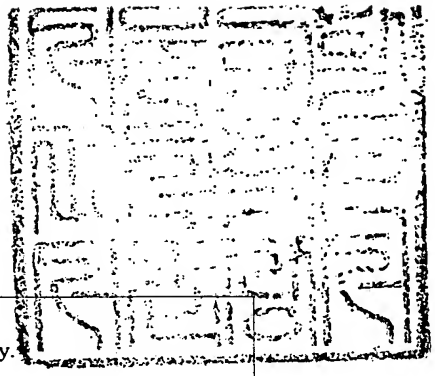

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$$\text{MAP} = \text{diastolic pressure} + \frac{\text{systolic pressure} - \text{diastolic pressure}}{3}.$$

mean circulatory filling p., a measure of the average (arterial and venous) pressure necessary to cause filling of the circulation with blood; it varies with blood volume and is directly proportional to the rate of venous return and thus to cardiac output.
negative p., a pressure less than that of the atmosphere.
occlusal p., pressure exerted on the occlusal surfaces of the teeth when the jaws are brought into apposition. Called also *biting p.*
oncotic p., the osmotic pressure due to the presence of colloids in a solution; in the case of plasma–interstitial fluid interaction, it is the force that tends to counterbalance the capillary blood pressure.
osmotic p., the pressure required to stop osmosis through a semipermeable membrane between a solution and pure solvent; it is proportional to the osmolality of the solution and also to other colligative properties of the solution, including freezing point depression, vapor pressure depression, and boiling point elevation. Symbol π.
osmotic p., effective, that part of the total osmotic pressure of a solution which governs the tendency of its solvent to pass through a semipermeable bounding membrane or across another boundary.
partial p., the pressure exerted by each of the components of a gas mixture.
perfusion p., the difference between the arterial and venous pressures through an organ or capillary bed.
pleural p., the pressure between the visceral pleura and the parietal pleura in the pleural cavity. Called also *intrapleural* or *intrathoracic p.*
positive p., pressure greater than that of the atmosphere.
positive end-expiratory p. (PEEP), a method of positive pressure ventilation used in conjunction with mechanical ventilation; pressure is maintained above the level of atmospheric pressure at the end of exhalation. This is achieved by preventing the complete release of gas during exhalation, usually by means of a valve within the circuit. The purpose is to increase the volume of gas remaining in the lungs at the end of expiration, thus reducing the shunting of blood through the lungs and improving gas exchange; done in acute respiratory failure to allow reduction of inspired O_2 concentrations. Cf. *continuous positive airway p.*
pulmonary artery wedge p. (PAWP), pulmonary capillary wedge p. (PCWP), intravascular pressure as measured by a catheter wedged into the distal pulmonary artery; it permits indirect measurement of the mean left atrial pressure.
pulse p., the difference between the systolic and diastolic pressures.
selection p., an effect produced by a given gene that determines the frequency of a given allele; it may be advantageous for survival *(positive selection pressure)* or disadvantageous *(negative selection pressure).*
solution p., the force that tends to bring into solution the molecules of a solid contained in the solvent.
systolic p., see *blood p.*
transpulmonary p., the pressure difference between the inner and outer surfaces of the lung, i.e., the pressure tending to inflate or deflate the lungs; equal to the difference between the alveolar pressure and the pleural pressure.
urethral p., the inward pressure exerted by the walls of the urethra, which must be counteracted in order for urine to flow through; see also under *profile.*
venous p., the blood pressure in a vein, such as central venous pressure or wedged hepatic vein pressure.
wedge p., blood pressure measured by a small catheter wedged into a vessel, occluding it; see *pulmonary capillary wedge p.* and *wedged hepatic vein p.*
wedged hepatic vein p., the venous pressure measured with a catheter wedged into the hepatic vein. The difference between wedged and free hepatic vein pressures is used to locate the site of obstruction in portal hypertension; it is elevated in that due to cirrhosis but low in cardiac ascites or portal vein thrombosis.

pre·su·bic·u·lum (pre″soo-bik′u-ləm) [TA] a modified six-layered cortex situated between the subiculum and the main part of the parahippocampal gyrus.

pre·sump·tive (pre-zump′tiv) referring to the expected fate of an embryonic part on the basis of established fate mapping.

pre·syl·vi·an (pre-sil′ve-ən) pertaining to the anterior or ascending branch of the sylvian fissure (sulcus lateralis).

pre·symp·tom (pre-simp′tom) an indication that is a forerunner of the actual symptoms of a condition.

pre·symp·to·mat·ic (pre″simp-to-mat′ik) existing before the appearance of symptoms.

pre·syn·ap·tic (pre″sĭ-nap′tik) situated before or proximal to a synapse, or occurring before the synapse is crossed.

pre·sys·to·le (pre-sis′to-le) the interval immediately preceding systole.

pre·sys·tol·ic (pre″sis-tol′ik) 1. pertaining to the beginning of systole. 2. occurring just before systole.

pre·tar·sal (pre-tahr′səl) anterior to the tarsus.

pre·tec·tal (pre-tek′təl) anterior to the tectum mesencephali.

pre·tec·tum (pre-tek′təm) pretectal area.

pre·throm·bot·ic (pre-throm-bot′ik) preceding the development of thrombosis.

pre·thy·roid (pre-thi′roid) anterior to the thyroid gland or thyroid cartilage.

pre·thy·roi·de·al (pre″thi-roi′de-əl) prethyroid.

pre·thy·roi·de·an (pre-thi-roi′de-ən) prethyroid.

pre·tu·ber·cu·lo·sis (pre″too-ber″ku-lo′sis) tuberculosis in an incipient and occult stage before any symptoms of the disease have appeared.

pre·ure·thri·tis (pre″u-re-thri′tis) inflammation of the vestibule of the vagina around the urethral orifice.

Prev·a·cid (prev′ə-sid) trademark for a preparation of lansoprazole.

prev AGT previous abnormality of glucose tolerance.

prev·a·lence (prev′ə-ləns) [L. *praevalēre* to prevail] [MeSH: Prevalence] the number of cases of a disease that are present in a population at a specified time, either at a point in time *(point p.)* or over a period of time *(period p.)*; when the term is unmodified, the former meaning is usually inferred. See *prevalence rate,* under *rate.* Cf. *incidence.*

pre·ven·tive (pre-ven′tiv) serving to avert the occurrence of.

pre·ven·tric·u·lus (pre″ven-trik′u-ləs) ostium cardiacum.

pre·ves·i·cal (pre-ves′ĭ-kəl) [*pre-* + *vesical*] anterior to the bladder.

pre·vi·a·ble (pre-vi′ə-bəl) not yet viable; said of a fetus incapable of extrauterine existence.

pre·vi·ta·min (pre-vi′tə-min) a precursor of a vitamin.
p. D_3, the immediate precursor to cholecalciferol, produced as a thermally labile intermediate upon irradiation of 7-dehydrocholesterol in the skin; at body temperature, it spontaneously rearranges to form cholecalciferol in approximately three days.

Pré·vost's law, sign (pra-vōz′) [Jean Louis *Prévost,* Swiss physician, 1838–1927] see under *law* and *sign.*

Pre·vo·tel·la (pre″vo-tel′ə) [André Romain Prévot, French microbiologist, 20th century] a genus of gram-negative, obligately anaerobic, moderately saccharolytic, bile-sensitive bacteria, consisting of nonmotile, non–spore-forming, pleomorphic rod-shaped organisms. They are normal inhabitants of the mucous membranes and are found especially in the oral cavity, colon, and vagina; some cause human infections. Included here are a number of species formerly included in the genus *Bacteroides.*
P. bi′via, a bile-sensitive, nonpigmented species that is moderately fermentative, found in the female genital tract and in the oral cavity, and isolated from infections of the urogenital tract and abdominal region and from breast abscesses. Called also *Bacteroides bivius.*
P. buc′cae, a nonpigmented species that is a normal inhabitant of the gingival crevice and has been isolated from chest drainage, blood, sinus aspirates, and peritoneal fluid; called also *Bacteroides buccae.*
P. cor′poris, a pigmented species that has been isolated from various clinical specimens; called also *Bacteroides corporis.*
P. denti′cola, a pigmented species that is a normal inhabitant of the gingival crevice and has been isolated from various clinical specimens; called also *Bacteroides denticola.*
P. di′siens, a nonpigmented, weakly fermentative species found in infections of the oral cavity and the female genital tract, and as part of the normal flora of the vagina and mouth; called also *Bacteroides disiens.*
P. heparinoly′tica, a nonpigmented species that has been isolated from infections of the oral cavity and respiratory tract and from the genital tract; called also *Bacteroides heparinolyticus.*
P. interme′dia, a weakly fermentative species isolated from the human gingival crevice and various clinical specimens. Called also *Bacteroides intermedius* and *B. melaninogenicus* subsp. *intermedius.*
P. melaninoge′nica, a coccoid species that produces a black hematin pigment, part of the normal flora of the mucous membranes. It is also an important pathogen in oral, lung, and brain abscesses and occurs in other mixed infections. Called also *Bacteroides melaninogenicus* and *B. melaninogenicus* subsp. *melaninogenicus.*
P. ora′lis, a nonpigmented, strongly fermentative species found principally in the gingival sulcus, which is occasionally associated with infections of the oral cavity and the respiratory and genital tracts; called also *Bacteroides oralis.*

physical c., the branch of chemistry that uses a quantitative approach, applying the concepts and laws of physics, to describe and understand chemical properties.
physiological c., biochemistry.
surface c., the study of forces acting at the surfaces of gases, liquids, or solid, or the interfaces between two states.
synthetic c., that branch of chemistry which deals with the building up of chemical compounds from simpler substances or from the elements.

chem(o)- [Gr. *chēmeia* alchemy] a combining form denoting relationship to chemistry, or to a chemical. Also, *chemi-*, *chemic(o)-*.

che·mo·at·trac·tant (ke″mo-ə-trak′tənt) a chemotactic factor that induces positive chemotaxis.

che·mo·au·to·troph (ke″mo-aw′to-trōf) a chemoautotrophic microorganism.

che·mo·au·to·troph·ic (ke″mo-aw″to-trōf′ik) [*chemo-* + *autotrophic*] requiring for growth only inorganic compounds with carbon dioxide as the sole source of carbon (autotrophic), and oxidizing inorganic chemical compounds as the source of energy; said of certain bacteria and protozoa. Cf. *photoautotrophic*.

che·mo·bi·ot·ic (ke″mo-bi-ot′ik) the combination of a chemotherapeutic agent and an antibiotic, as of one or more of the sulfonamide compounds with penicillin.

che·mo·cau·tery (ke″mo-kaw′tər-e) destruction of tissue by application of a caustic chemical substance. Called also *chemical cautery*.

che·mo·ce·pha·lia (ke″mo-sə-fa′le-ə) chamaecephaly.

che·mo·ceph·a·ly (ke″mo-sef′ə-le) chamaecephaly.

che·mo·cep·tor (ke′mo-sep-tor) chemoreceptor.

che·mo·co·ag·u·la·tion (ke″mo-ko-ag″u-la′shən) coagulation or destruction of tissue by the application of chemicals.

che·mo·dec·to·ma (ke″mo-dek-to′mə) [*chemo-* + *dektos* to be received or accepted + *-oma*] any benign, chromaffin-negative tumor of the chemoreceptor system; the most common types are the *carotid body tumor*, the *glomus jugulare tumor*, and the *glomus vagale tumor*. Called also *nonchromaffin paraganglioma*.

che·mo·dif·fer·en·ti·a·tion (ke″mo-dif″ər-ən-she-a′shən) the invisible point of decision which foreruns and controls the actual differentiation of cells into the rudimentary organs of the embryo.

che·mo·dy·ne·sis (ke″mo-di′nə-sis) the initiation of cytoplasmic streaming in plant cells by chemicals.

che·mo·em·bo·li·za·tion (ke″mo-em″bo-lĭ-za′shən) percutaneous introduction of a substance to occlude a vessel in combination with a chemotherapeutic agent, used in the treatment of cancer to deliver sustained therapeutic levels of the agent to a tumor.

che·mo·en·do·crine (ke″mo-en′do-krin) [*chemo-* + *endocrine*] chemohormonal.

che·mo·het·ero·troph (ke″mo-het′ər-o-trōf″) a chemoheterotrophic organism.

che·mo·het·ero·troph·ic (ke″mo-het″ər-o-trof′ik) heterotrophic; requiring preformed organic compounds as a source of carbon and oxidizing organic compounds as a source of energy.

che·mo·hor·mo·nal (ke″mo-hor-mo′nəl) pertaining to drugs having hormone activity.

che·mo·im·mu·nol·o·gy (ke″mo-im-u-nol′ə-je) immunochemistry.

che·mo·kine (ke′mo-kīn) any of a group of low molecular weight cytokines, such as interleukin-8, identified on the basis of their ability to induce chemotaxis or chemokinesis in leukocytes (or in particular populations of leukocytes) in inflammation, the group now divided into four subgroups on the basis of genetic, structural, and functional criteria. They function as regulators of the immune system and may also play roles in the circulatory and central nervous systems.

che·mo·ki·ne·sis (ke″mo-kī-ne′sis) [*chemo-* + *-kinesis*] increased nondirectional activity of cells due to presence of a chemical substance. Cf. *chemotaxis*.

che·mo·ki·net·ic (ke″mo-kī-net′ik) pertaining to or exhibiting chemokinesis.

che·mo·litho·troph (ke″mo-lith′o-trōf) a chemolithotrophic organism.

che·mo·litho·troph·ic (ke″mo-lith″o-trof′ik) chemoautotrophic; utilizing carbon dioxide as the sole source of carbon and deriving energy from the oxidation of inorganic compounds.

che·mo·lu·mi·nes·cence (ke″mo-loo″mĭ-nes′əns) chemiluminescence.

che·mol·y·sis (ke-mol′ĭ-sis) [*chemo-* + *-lysis*] chemical decomposition.

che·mo·mor·pho·sis (ke″mo-mor-fo′sis) [*chemo-* + *morph*] change of form or developmental stage due to chemical action.

che·mo·nu·cle·ol·y·sis (ke″mo-noo″kle-ol′ə-sis) [*chemo-* + *nucl* + *lysis*] dissolution of the nucleus pulposus of an intervertebral disk by injection of a proteolytic agent such as chymopapain, especially in the treatment of herniation of an intervertebral disk (see under *herniation*).

che·mo·or·gano·troph (ke″mo-or′gə-no-trōf″) a chemoorganotrophic organism.

che·mo·or·gano·troph·ic (ke″mo-or″gə-no-trof′ik) heterotrophic; requiring preformed organic compounds as a source of carbon and oxidizing organic compounds as a source of energy; said of bacteria.

che·mo·pal·li·dec·tomy (ke″mo-pal″ĭ-dek′tə-me) [*chemo-* + *pallidectomy*] destruction of a portion of the globus pallidus by the introduction of a chemical agent.

che·mo·pal·li·do·thal·a·mec·to·my (ke″mo-pal″ĭ-do-thal″ə-mek′tə-me) destruction of a portion of the globus pallidus and thalamus by the introduction of a chemical agent.

che·mo·phar·ma·co·dy·nam·ic (ke″mo-fahr″mə-ko-di-nam′ik) denoting the relationship between chemical constitution and biologic or pharmacologic activity.

che·mo·phys·i·ol·o·gy (ke″mo-fiz″e-ol′ə-je) biochemistry.

che·mo·pre·ven·tion (ke″mo-pre-ven′shən) [MeSH: Chemoprevention] chemoprophylaxis.

che·mo·pro·phy·lax·is (ke″mo-pro″fə-lak′sis) [*chemo-* + *prophylaxis*] use of a chemotherapeutic agent as a means of preventing development of a specific disease. Called also *chemoprevention*, *chemical prophylaxis*, and *drug prophylaxis*.
primary c., prophylactic use of a chemotherapeutic agent before infection has occurred in an individual.
secondary c., prophylactic use of a chemotherapeutic agent in an individual after infection has occurred (with *Mycobacterium tuberculosis*, for example) but before disease has become manifest.

che·mo·pro·tec·tant (ke″mo-pro-tek′tənt) 1. providing protection against the toxic effects of chemotherapeutic agents. 2. an agent that provides protection against the toxic effects of chemotherapeutic agents.

che·mo·psy·chi·a·try (ke″mo-si-ki′ə-tre) the use of drugs in the treatment of mental and emotional disorders; psychopharmacology.

che·mo·ra·dio·ther·a·py (ke″mo-ra″de-o-ther′ə-pe) [*chemo-* + *radiotherapy*] combined modality therapy using chemotherapy and radiotherapy, designed to reduce the need for surgery by maximizing the interaction between the radiation and the therapeutic agent or agents.

che·mo·re·cep·tion (ke″mo-re-sep′shən) the process of being sensitive to or perceiving chemical stimuli in the surrounding medium.

che·mo·re·cep·tor (ke″mo-re-sep′tər) [*chemo-* + *receptor*] [MeSH: Chemoreceptors] 1. a receptor adapted for excitation by chemical substances, e.g., olfactory and gustatory receptors. 2. a sense organ such as the carotid body, the aortic bodies, or the glomus jugulare, which is sensitive to chemical changes in the blood stream, especially reduced oxygen content, and reflexly increases both respiration and blood pressure. See also *receptor*, def. 2 and *chemoreceptor system*, under *system*. 3. a supposed group of atoms in cell protoplasm having the power of fixing chemicals, in the same way as bacterial poisons are fixed. Called also *chemoceptor*.

che·mo·re·sis·tance (ke″mo-re-zis′təns) specific resistance acquired by cells to the action of chemicals.

che·mo·sen·si·tive (ke″mo-sen′sĭ-tiv) sensitive to changes in chemical composition of the environment.

che·mo·sen·sory (ke″mo-sen′sər-e) relating to the perception of chemical substances, as in odor detection.

che·mo·se·ro·ther·a·py (ke″mo-se″ro-ther′ə-pe) the treatment of disease with both drugs and serum.

che·mo·sis (ke-mo′sis) [Gr. *chēmōsis*] excessive edema of the ocular conjunctiva.

chem·os·mo·sis (ke″mos-mo′sis) chemiosmosis.

chem·os·mot·ic (ke″mos-mot′ik) chemiosmotic.

che·mo·sorp·tion (kem″o-sorp′shən) chemisorption.

che·mo·sphere (ke′mo-sfēr) the layer of the upper atmosphere where photochemical reactions become important (30–80 km).

che·mo·stat (ke′mo-stat) an apparatus in which the environment

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Progress in Cancer Chemoprevention

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ABSTRACT: **More than 40 promising agents and agent combinations are being evaluated clinically as chemopreventive drugs for major cancer targets. A few have been in vanguard, large-scale intervention trials—for example, the studies of tamoxifen and fenretinide in breast, 13-*cis*-retinoic acid in head and neck, vitamin E and selenium in prostate, and calcium in colon. These and other agents are currently in phase II chemoprevention trials to establish the scope of their chemopreventive efficacy and to develop intermediate biomarkers as surrogate end points for cancer incidence in future studies. In this group are fenretinide, 2-difluoromethylornithine, and oltipraz. Nonsteroidal anti-inflammatories (NSAID) are also in this group because of their colon cancer chemopreventive effects in clinical intervention, epidemiological, and animal studies. New agents are continually considered for development as chemopreventive drugs. Preventive strategies with antiandrogens are evolving for prostate cancer. Anti-inflammatories that selectively inhibit inducible cyclooxygenase (COX)-2 are being investigated in colon as alternatives to the NSAID, which inhibit both COX-1 and COX-2 and derive their toxicity from COX-1 inhibition. Newer retinoids with reduced toxicity, increased efficacy, or both (e.g., 9-*cis*-retinoic acid) are being investigated. Promising chemopreventive drugs are also being developed from dietary substances (e.g., green and black tea polyphenols, soy isoflavones, curcumin, phenethyl isothiocyanate, sulforaphane, lycopene, indole-3-carbinol, perillyl alcohol). Basic and translational research necessary to progress in chemopreventive agent development includes, for example, (1) molecular and genomic biomarkers that can be used for risk assessment and as surrogate end points in clinical studies, (2) animal carcinogenesis models that mimic human disease (including transgenic and gene knockout mice), and (3) novel agent treatment regimens (e.g., local delivery to cancer targets, agent combinations, and pharmacodynamically guided dosing).**

Cancer chemoprevention is defined as the use of specific chemical compounds to prevent, inhibit, or reverse carcinogenesis.[1,2] In many major cancer targets, human cancer development requires 20–40 years or more,[1] and the scope of chemopreven-

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Genetic Alterations in Mouse Lung Tumors: Implications for Cancer Chemoprevention

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Abstract Specific genetic alterations affecting known tumor suppressor genes and proto-oncogenes occur during mouse lung tumorigenesis. These include mutational activation of the K-*ras* gene, commonly seen at a frequency of about 80% in both spontaneously occurring and chemically induced adenomas and adenocarcinomas of the lung, suggesting that it is an early event that persists into malignancy. Allelic loss of the p16 tumor suppressor gene also is a frequent event, occurring in about 50% of mouse lung adenocarcinomas, but rarely in lung adenomas, suggesting that it may play a role in malignant conversion or progression of lung tumors. Other genetic alterations detected in mouse lung tumors include reduced expression of Rb and p16, and increased c-*myc* expression. Alterations of these genes are also common in the genesis of human lung cancer. Genetic linkage analysis to identify human lung cancer susceptibility genes is difficult due to the genetic heterogeneity and exposure to environmental risk factors. The mouse lung tumor model has become a valuable alternative for identifying such genes. Recently, loci responsible for mouse lung tumor susceptibility have been mapped to chromosomes 6, 9, 17, and 19, while those linked to lung tumor resistance have been mapped to chromosomes 4, 11, 12, and 18. Known candidate susceptibility or resistance genes include the K-*ras* proto-oncogene on chromosome 6, and the p16 tumor supressor gene on chromosome 4. With evidence of considerable overlap between the genetic alterations that underlie human and mouse lung tumorigenesis, the mouse lung tumor model has been expanded to include pre-clinical screening of chemopreventive agents against human lung cancer. Studies on the modulation of genetic defects in mouse lung tumors by known and potential chemopreventive agents should further the goal of developing an effective prevention and treatment of lung cancer. J. Cell. Biochem. Suppls. 28/29:49–63.

Key words: mouse lung; genetic alteration; aberrant gene expression; surrogate endpoint biomarkers; cancer chemoprevention

Lung cancer is the leading cause of cancer mortality in both males and females in developed countries [1]. Epidemiological studies have indicated that approximately 85% of all lung cancer deaths in the United States are associated with tobacco smoking [2]. Relative risk for lung cancer is increased in smokers at least 13-fold and in passive smokers by 1.5-fold, with a linear relationship between the number of cigarettes smoked and lung cancer risk [3,4]. Approximately 50 of the chemicals in cigarette smoke, including polyaromatic hydrocarbons, nitrosamines, and aromatic amines, have been shown to be mutagenic or carcinogenic [5]. Additional environmental and occupational risk factors for lung cancer include exposure to asbestos, arsenic, chromium, nickel, and radon [6]. The mouse lung tumor bioassay was developed more than 50 years ago to identify potential lung carcinogens, and has been instrumental in demonstrating the carcinogenicity of a wide range of chemicals [7].

Lung cancer, like other types of cancer, develops as a multistage process involving the accumulation of genetic alterations that affect key proto-oncogenes and tumor suppressor genes. Many of the known changes are common to both human and mouse lung tumors. Figure 1 shows the genetic alterations detected during mouse lung tumorigenesis. To date, the most common alterations in mouse lung tumors have affected genes associated with numerous cancer types. Mutation of the K-*ras* proto-oncogene

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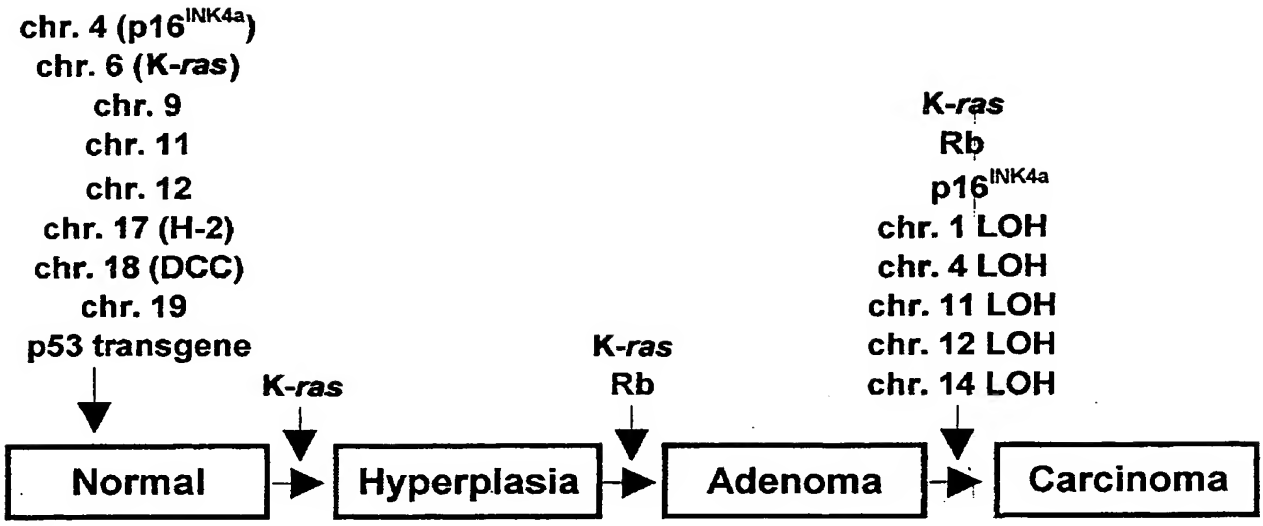

Fig. 1. Genetic alterations found during mouse lung tumorigenesis.

is seen in about 80% of mouse lung adenomas and adenocarcinomas and is the principal candidate for a major susceptibility gene on chromosome 6 [8,9]. Other frequent alterations affect tumor suppressor genes p16 and p53, which have been associated with specific stages of both human and mouse lung tumorigenesis [10,11]. Aberrant expression of c-*myc*, Rb and p16 gene also have been found in mouse lung tumorigenesis [12] (Liu et al., unpublished data). Finally, newly developed screening methods have revealed numerous genetic changes, suggesting that additional unidentified genes may also contribute to mouse lung tumorigenesis (Lin et al., unpublished data).

Over the past decade, a number of genes predisposing to the development of specific types of cancer have been identified [13]. Similarly, there is evidence that the susceptibility of the human population to different forms of lung cancer follows a pattern of autosomal dominant Mendelian inheritance [14–18]. However, the pervasiveness of lung carcinogens in our environment has made it difficult to accurately identify familial clusters of lung cancer patients necessary to identify predisposing genes. The use of mouse models imparts control over environmental factors that confound human studies on the genetics of lung cancer. Inbred mouse strains are variable in genotype, as well as in susceptibility to lung cancer, ranging from the very susceptible A/J strain to the very resistant C57BL/6J strain [19]. Genetic studies have taken advantage of these strain differences to map lung tumor susceptibility and resistance genes to specific chromosome locations [20]. Thus far, linkage has been demonstrated for loci on chromosomes 4, 6, 9, 11, 12, 17, 18, and 19 [9, 21–29]. Knowing which genes predispose or underlie the development of mouse lung cancer is of considerable interest to further understanding of human disease. A number of candidate susceptibility and resistance loci now exist based on their chromosomal location relative to the regions of strong linkage, and in some cases, also based on prior demonstrated involvement in mouse lung tumorigenesis. Chemopreventive strategies may use molecular changes that control the genesis of lung cancer as targets or intermediate endpoint biomarkers. Progress toward understanding these changes is reviewed below.

SUSCEPTIBILITY OF MOUSE STRAINS TO CHEMICAL LUNG TUMOR INDUCTION

Although the majority of lung cancer cases are associated with cigarette smoking and environmental exposure, increasing evidence suggests that individuals differ in their susceptibility to environmental factors. An increased familial risk for lung cancer has been observed within lung cancer probands [14]. Further segregation analyses provided evidence that susceptibility of the human population to different forms of lung cancer follows a pattern of autosomal dominant Mendelian inheritance [14–18]. However, there have been no reports on localiza-

tion and identification of human lung cancer susceptibility gene(s).

Genetic differences between mouse strains are analogous to genetic differences within the human population, making the mouse lung system an excellent tool for studying genetic components underlying tumor development and susceptibility [30]. Inbred mouse strains show widely different susceptibilities to both spontaneously occurring and chemically induced lung tumor formation [31]. This susceptibility is intrinsic to the lung itself, as shown by classic experiments involving lung explants from sensitive and resistant mice, which showed that tumors developed after carcinogen treatment only in lungs of the sensitive mouse strain [32, 33]. Matings of sensitive A/J and resistant C57BL/6J mice produce F_1 and F_2 offspring which have intermediate sensitivity to tumor induction, indicating that this phenotype is conferred by more than one gene [34]. The production of recombinant inbred (RI) lines of A/J and C57BL/6J mice and subsequent analysis of their sensitivities to tumorigenesis originally suggested that three genes, one major and two minor, were involved in determining the sensitivity to mouse lung tumor development [34]. Subsequent linkage studies were conducted to identify pulmonary adenoma susceptibility (*Pas*) and pulmonary adenoma resistance (*Par*) loci, with tumor multiplicity and size used as quantitative traits. These results have revealed the polygenic nature of the predisposition to tumor induction of the mouse lung. Listed in Table I and outlined below are the quantitative trait loci (QTL) that have been mapped to lung tumor susceptibility/resistance in various mouse crosses.

Pas1

A major susceptibility locus was mapped to distal chromosome 6 in (A/J x C3H/HeJ) F_2 mice, and was termed the *Pas1* locus. This locus produced a maximum logarithm of the likelihood ratio (LOD) score of 9 and accounted for approximately 45% of the observed phenotypic variance [9]. A LOD score of 3 or greater is considered significant for linkage. Corroborating results were obtained in comprehensive linkage studies using (A/J x C57BL/6J) F_2 (60% of variance), (A/J x C57BL/6J) x C57BL/6J (16% of variance), (A/J x *M. spretus*) x C57BL/6J (34% of variance), and AxB and BxA RI mice (51% of variance) [21–23, 35]. The QTL for *Pas1* showed tightest linkage at the locus of the K-*ras* gene, which became the principal candidate gene for *Pas1* based on the understanding that K-*ras* gene activation is an early event often found in

TABLE I. Mouse Lung Tumor Quantitative Trait Loci (QTL)

QTL	Chr	Cross[a]	Variance (%)	Candidates	References
Pas 1	6	A × B & B × A RI	51	K-*ras*	[23]
		(A/J × C3H/He)F_2	40	K-*ras*	[9]
		(A/J × B6)F_2	60	K-*ras*	[21]
		(A/J × B6) × B6	16	K-*ras*	[22]
		(A/J × *M. spretus*) × B6	40	K-*ras*	[35]
Pas 2	17	(A/J × B6)F_2	7	TNF α/β	[21]
		A × B & B × A RI	29	TNF α/β	[23]
Pas 3	19	(A/J × B6) × B6	3		[22]
		(A/J × B6)F_2	2		[21]
		A × B & B × A RI	26		[23]
Pas 4	9	(A/J × B6)F_2	4		[21]
Pas 5	10	A × B & B × A RI	22		[23]
Par 1	11	(A/J × *M. spretus*) × B6	15		[26]
Par 2	18	(A/J × BALB/c) × A/J	38	DCC	[27]
		(A × BALB/c)F_2	~50	DCC	[25]
Par 3	4	(A × BALB/c)F_2	~10	$p16^{INK4a}$	[24]
Par 4	12	SM × A RI			[29]
Sluc 1	19(D19MIT9)	(OcB-9 × 020)F_2			[28]
Sluc 2	2(D2MIT56)	(OcB-9 × 020)F_2			[28]
Sluc 3	6(D6MIT218)	(OcB-9 × 020)F_2		TNF R1	[28]
Sluc 4	11(D11MIT15)	(OcB-9 × 020)F_2		p53	[28]

[a]B6 = C57BL/6; B = C57BL/6; A = A/J.

both spontaneously occurring and chemically induced mouse lung tumors, and that polymorphisms detected in K-*ras* promoter and enhancer regions in different mouse strains correlate with their susceptibility to chemical induction of lung tumors [8,36]. Also, these polymorphisms seem to be responsible for the observed allele-specific expression of the K-*ras* allele in hybrid mice, which leads to allele-specific activation of the K-*ras* gene [19,37]. Finally, genetic linkage analyses indicate a major locus at this location only when parental mice have distinct K-*ras* genotypes; for example, studies on (A/J x BALB/cByJ) x A/J and (A/JO1aHsd x BALB/cO1aHsd) F_2 mice showed no linkage between lung tumor formation and *Pas1* [25,27], indicating that BALB/c and A/J are genetically alike at the *Pas1* locus. Both of these strains possess the same K-*ras* variant.

Pas2, Pas3, and *Pas4*

Loci shown to positively modulate the effect of *Pas1* were mapped to chromosomes 9, 17, and 19 [21,22]. Linkage to the site of the putative *Pas2* locus on chromosome 17 was observed in (A/J x C57BL/6J) F_2. This locus accounted for 7% of the total variance in phenotype. The location of the *Pas2* locus is homologous to human chromosome 6p21; possible candidates at this location are the genes for tumor necrosis factor (TNF) α and β. Similarly, linkages to lung tumor susceptibility were also seen at markers on chromosome 19 (*Pas3*), accounting for 3% of the phenotypic variation in a study on (A/J x C57BL/6J) x C57BL/6J mice, and 2% of the explained phenotypic variation when (A/J x C57BL/6J) F_2 mice were used. In this latter study, suggestive linkage to a locus on chromosome 9 (*Pas4*) was determined to explain 4% of the total phenotypic variance [21].

Par1

A lung tumor resistance locus, *Par1*, was recently mapped in (A/J x *M. spretus)* x C57BL/6J mice to chromosome 11 overlapping the retinoic acid receptor-α (*Rara)* gene locus [26]. Contributed by the *M. spretus* allele, *Par1* gave a maximum LOD score of 5.3 and accounted for 23% of phenotypic variance when co-expressed with the highly penetrant *Pas1* allele of the A/J strain. In mice carrying the *M. spretus* instead of the A/J allele of the *Pas1* gene, the resistant effect of *Par1* on tumor incidence, multiplicity and volume was lessened by about one-half. Thus, *Par1* behaves like a modulator of *Pas1*, to some degree subduing the dominant effect of *Pas1* on lung tumorigenesis [26].

Par2

Linkage studies in (A/J x BALB/cByJ) x A/J and (A/JO1aHsd x BALB/cO1aHsd) F_2 mice revealed significant linkage on chromosome 18 at microsatellite marker D18MIT103. A LOD score of 12.2 was reported at this locus, with a phenotypic variance of 38% for resistance to tumor induction [27]. This locus was termed *Par2*. In our analysis of (A/JO1aHsd x BALB/cO1aHsd) F_2 mice, *Par2* had a significant linkage to lung tumor resistance and produced a maximum LOD score of 11 [25]. The greatest linkage occurred at the site of the *DCC* tumor suppressor gene [25,27]. The *DCC* gene was identified on human chromosome 18q21 as a target of somatic mutation and allelic loss in colorectal carcinomas [38]. Since its identification, many studies have shown that its loss is common to several other types of cancer including those of breast, prostate, esophagus, endometrium, pancreas, stomach, and brain [39,40]. However, the role of *DCC* as a tumor suppressor is still in question since *DCC* deficient mice did not develop any tumors. This gene codes for a transmembrane protein comprised of four immunoglobulin-like and six fibronectin Type III-like domains. Recently, *DCC* was shown to function in the nervous system as a neurin receptor or receptor component that mediates neurin-directed axon outgrowth [41]. Of potential significance to lung cancer, *DCC* has been shown to suppress the malignant phenotype of transformed human epithelial cells [42]. The human and mouse *DCC* proteins share 96% identity, and their genes are tightly linked to two other candidate tumor suppressor genes, with human homologues named Deleted in Pancreatic Cancer 4 (DPC4) and MADR2/JV18–1. Recently, we observed no sequence polymorphisms in the *DCC* gene between the A/J allele and BALB/cJ allele, or difference in allele-specific expression of the *DCC* gene, that suggests against its inclusion as a candidate lung tumor resistant gene (Lin et al., unpublished data). Similar observations have also been made for DPC4 and MADR2/JV18–1 [25].

Par3

We also observed linkages to susceptibility on chromosome 4 (D4MIT77) (LOD score = 3.0) using (A/JO1aHsd x BALB/cO1aHsd) F_2 mice [24]. *Par3* seems to have a stronger resistance to lung tumor induction when co-expressed with the A/J allele of *Par2* [24, 43]. Linkage on chromosome 4 was strongest at a marker recombinationally inseparable from the p16 tumor suppressor gene locus; the BALB/cJ allele at this locus is associated with sensitivity to lung tumor formation. p16 has been shown to specifically inhibit serine/threonine protein kinase activity of cyclin D-dependent kineses CDK4 and CDK6 [44]. The principal target of these kinases is the retinoblastoma protein (pRb). When phosphorylated by CDK4 and/or CDK6, pRb is inactivated and rendered incapable of maintaining its inhibitory sequestration of the E2F family of transcription factors. Upon release, these transcription factors activate the transcription of genes important in cell growth, such as dihydrofolate reductase, thymidine kinase, and cyclin A and E [45]. Overexpression of members of the E2F family have been shown to override p16-induced cell cycle arrest [45]. p16 and pRb are both negative regulators of the passage of cells through the G1 phase of the cell cycle; inactivation of either is common to several types of cancer [46].

There is evidence to suggest that the p16 gene is a candidate for *Par3*. Two variants of p16 that differ at amino acids 18 and 51 were shown to exist in mice [43]. The A/J and BALB/c strains represent the two groups of variant strains. These observations suggest that the BALB/c variant of p16 may confer resistance to lung tumorigenesis.

Par4

A locus conferring resistance to urethane-induced lung tumorigenesis was mapped to chromosome 12 (LOD score 6.4) using SM x A RI strains of mice [29]. One potential candidate gene for *Par4* is protein kinase Cη which is expressed only in skin and lung tissues.

Sluc1, Sluc2, Sluc3, and Sluc4

Complex interactions between QTLs on chromosomes 19 (distinct from *Pas3*) and chromosome 2, and between loci on chromosome 6 (distinct from *Pas1*) and chromosome 11 (distinct from *Par1*), were suggested to influence lung tumor size or rate of growth in (OcB-9 x O20) F_2 mice [28]. These loci were termed Susceptibility to lung cancer 1 (*Sluc1*), *Sluc2*, *Sluc3*, and *Sluc4*, respectively. OcB-9 is a recombinant congenic strain that shares 87.5% of its genetic identity with the O20 inbred strain, and 12.5% with strain B10.O20. This study used multiple-QTL models (MQM) mapping allowing for interactions between QTLs. It was observed that within an interaction, the affect on tumor size depended on the genotype of each locus. For example, the affect of *Sluc1* on tumor size was large when *Sluc2* was homozygous for O20, but small when *Sluc2* was homozygous for B10.O20. The effect of *Sluc3* on tumor size was large when *Sluc4* was homozygous for the O20 allele and small when *Sluc4* was homozygous for the B10.O20 allele. Also, susceptibility to large tumors was seen when *Sluc1* and *Sluc2* were both homozygous for O20, but significant resistance was seen when *Sluc1* was homozygous BI0.O20 and *Sluc2* was homozygous O20. Similarly, susceptibility to large tumors was significant when *Sluc3* was homozygous O20 and *Sluc4* was homozygous B10.O20. Significant linkage with very small tumors occurred when both *Sluc3* and *Sluc4* were homozygous for B10.O20. Interestingly, the interacting loci *Sluc3* and *Sluc4* map to the same approximate locations as the genes for TNF receptor 1 and the p53 tumor suppressor, respectively, whose functions could conceivably interact to affect tumor volume.

ONCOGENES

K-*ras* mutations detected during mouse lung tumorigenesis tend to occur early and persist into malignancy [8]. The mutation spectra in the K-*ras* gene of these tumors induced by different carcinogens are clearly distinct and consistent with the expected mutagenic specificity of the carcinogens [8]. As shown in Table II, we observed an almost exclusive occurrence of activated mutations in the A/J K-*ras* allele in lung tumors of F1 hybrid offspring produced from crosses between strains of mice that are susceptible and resistant to lung tumor formation [36]. DNA sequence variations between inbred mouse strains have been detected in the second intron [19], 5'-promoter region (Zhang et al., unpublished data) and 3'-untranslated region (UTR) [47] of the K-*ras* gene, which may contribute to the constitutively higher expression of the A/J allele relative to the C3H allele.

TABLE II. Allele-Specific Expression and Localization of the K-*ras* Oncogene Detected in hybrid Mouse Lung Tumors*

Treatment	F_1 hybrids	Activated K-*ras* gene	Allele-specific expression: Alleles	Allele-specific expression: ↑ (fold)	Allele-specific location: Others	Allele-specific location: A/J or BALB/c	References
None	(C3H/HeJ × A/J)F_1	20	A/J > C3H	ND	2	18	[19, 85]
NNK	(C3H/HeJ × A/J)F_1	7	A/J > C3H	2–50	0	7	[19, 85]
VC	(C3H/HeJ × A/J)F_1	13	A/J > C3H	2–50	0	13	[19, 85]
NNK	(A/J × C3H/HeJ)F_1	19	A/J > C3H	2–50	0	19	[19, 85]
VC	(A/J × C3H/HeJ)F_1	15	A/J > C3H	2–50	0	14	[19, 81]
DMN	(C3H/HeJ × A/J)F_1	15	A/J > C3H	2–50	0	14	[37]
B(*a*)P	(C3H/HeJ × A/J)F_1	15	A/J > C3H	2–50	0	15	[37]
NNK	(A/J × TSGp53)F_1	38	A/J > TSGp53	10–20	0	38	[47]
MNU	(A/J × *M. Spretus*)F_1	12	A/J > *M. Spretus*	1.7–12	0	12	[51]
VC	(C57BL/6J × BALB/cJ)F_1	18	N.D.	N.D.	3	15	[49]
AFB_1	(A/J × C3H/HeJ)F_1	76	N.D.	N.D.	3	73	[50]
ENU	RCS	134	N.D.	N.D.	1/71	13/63	[86]

*N.D., not determined. NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; VC, vinyl chloride; DMN, dimethylnitrosamine; B(*a*)P, benzo(*a*)pyrene; MNU, *N*-methylnitrosourea; AFB_1, aflatoxin B_1; ENU, *N*-ethylnitrosourea; RCS, recombinant congenic strains.

In fact, differential protein-binding patterns were observed in gel mobility-shift experiments between the duplicated 37 bp sequence of the K^R allele (K-*ras* allele identical to that of the resistant C3H/HeJ strain) and the single 37 bp sequence of the K^S and K^I alleles (K-*ras* alleles identical to that of the susceptible A/J strain) [19]. DNase I footprinting assays revealed protein binding sites in the second intron of the K-*ras* gene, which corresponded to the tandem repeat sequences. In a separate study, another protein-binding site located downstream (nucleotides 463–509) from the tandem repeat region was identified and shown by DNase I footprinting to be a protein binding site in the second intron [48]. Southwestern blot analysis indicated that these two repetitive regions could be involved in binding with the same regulatory complex. Furthermore, gel mobility-shift studies showed differential protein-binding patterns between the K^R allele and the K^S/K^I allele. These results suggest that the repetitive sequences in the second intron could play a role in differential transcriptional regulation of the K-*ras* gene.

The K^S allele was also expressed at significantly higher levels than K^R alleles in lung tissue from hybrid mice (Table II) [19,37,47]. For example, K^S was 1.4 to 2 times higher than K^R in lung tissues from 4–8-week-old untreated (C3H/HeJ X A/J) F_1 or (A/J X C3H/HeJ) F_1 mice, and 2 to 12 times higher in (A/J X C57BL/6J) F_1 hybrid mouse lung tissues harvested over a 20-week period. Furthermore, oncogenic K-*ras* from the A/J parent is expressed 2–50 times over the normal level of C3H allele in lung tumors (Table II). As alluded to above, lung tumors from (C3H/HeJ X A/J) F_1 or (A/J X C3H/HeJ) F_1 mice nearly always display activating mutations of the K^S allele. Similar results have been observed in lung tumors from (A/J X TSGp53) F_1, (A/J X *M. Spretus*) F_1, and (C57BL/6J x BALB/cJ) F_1 mice (Table II) [35,49,50]. The chloramphenicol acetyltransferase (CAT) assay was used to compare the transcription-stimulating activity of K-*ras* intron 2 putative enhancer regions of K^R and K^S alleles using the nontumorigenic C10 cell line derived from normal alveolar Type II cells. This analysis demonstrated that enhancer activity of K^S was 2.4–9.1-fold higher than that of K^R and the *M. spretus* K-*ras* allele, irrespective of orientation [48, 51]. *Mus Spretus*, wild strain that is resistant to lung tumor formation, possesses a variant of K-*ras* with polymorphisms distinct from those of K^S, K^I, and K^R alleles. Observed sequence variations in the intron 2 putative enhancer region among different strains of the K-*ras* gene may contribute to observed differences in the levels of K-*ras* expression among different mouse strains. These findings suggest that mutational activation of the more highly expressed K-*ras* allele may provide a selective

advantage during mouse hybrid lung carcinogenesis.

As shown in Table III, overexpression of c-*myc* proto-oncogene also has been observed at a high frequency in urethane-induced mouse lung tumors [12]. Northern blot analysis showed a 3–5-fold increase in c-*myc* transcripts in 8 of 11 (A/J X C3H/HeJ) F_1 and 4 of 5 BALB/c lung tumors. The c-*myc* proto-oncogene is an important regulator of cell proliferation and apoptosis, and its constitutive expression enforces proliferation and sensitizes cells to apoptosis [52]. Recent evidence suggests that c-*myc* appears to function as both activator and repressor of growth antagonistic genes [53]. Also, c-*myc* overexpression prevents growth arrest induced by p16 and can bypass p16/Rb enforced growth arrest. Thus, c-*myc* is thought to function downstream of p16/Rb, perhaps mimicking the effect of an inactive p16/Rb pathway [54]. Table III summarizes differentially expressed genes detected in mouse lung tumors.

Various other genes have shown increased levels of expression in mouse lung tumors. For example, Re, et al. reported a 3–5-fold increase in the mRNA level of the pulmonary surfactant protein-A (SP-A) gene in all tumors examined (11 (A/J X C3H/HeJ) F_1, 5 BALB/c, and 9 A/J) (Table III) [12]. The authors concluded that all tumors examined were derived from either alveolar type II or Clara cells on the basis that SP-A and other surfactants, including SP-B and SP-D, are selectively expressed by these cell types.

TUMOR SUPPRESSOR GENES

Allelotype and LOH studies have been conducted to identify regions of frequent allelic loss in lung tumors of various F_1 hybrid mouse strains in order to localize important tumor suppressor genes [10,11,55–58]. As shown in Table IV, the loci most commonly affected by allelic loss were shown to reside on chromosome 4. Deletion mapping studies involving lung adenocarcinomas of (A/J x C3H/HeJ) F_1, (C3H/HeJ x A/J) F_1, (BALB/cJ x DBA/2J) F_1, and (C57BL/6J x C3H/HeJ) F_1 mice implicated two distinct lung tumor suppressor loci on this chromosome [10, 56–58]. One locus was mapped to the p15 and p16 genes on mid-chromosome 4, deletions of which occurred in about half of the adenocarcinomas examined [10]. The p15 and p16 genes were later shown to be homozygously codeleted in 12 of 16 (75%) lung tumor cell lines derived from inbred mouse strains [59]. Both of these studies demonstrated narrow regions of deletion, which strongly suggested that p16, and perhaps p15, inactivation contributes to mouse lung tumorigenesis. In comparison, human p16 and p15 have been the target of deletion at a similar frequency in human non-small cell carcinomas [60]. It is interesting that the p19ARF gene, which overlaps with p16, is also commonly deleted along with p16 in both human and mouse lung cancer, suggesting that this narrow region of the genome could harbor more than one lung tumor suppressor gene [61–64].

We recently demonstrated the existence of two variants of the mouse p16 gene [43]. Observed sequence polymorphisms constituted three amino acid differences; one at position 18 and another at position 51 of exon 2. Most strains encode a histidine (CAT) at position 18 and a valine (GTA) at position 51; however, six of the strains (BALB/c, O20, C3H/HeJ, C3H/21BG, CBA/J, and PL/J) code for proline (CCT) and isoleucine (ATA) at these positions, respectively. The p19ARF gene shares the p16 locus by utilizing the same second exon in an alter-

TABLE III. Aberrant Levels of Gene Expression in Primary Mouse Lung Tumors*

Gene	Alteration		References
	mRNA	Protein	
Rb	Decreased	Decreased	[12, 65] (Liu et al., unpublished data)
p16	Decreased	Decreased	[65] (Liu et al., unpublished data)
Growth arrest-specific 3	Decreased	N.D.	[12]
Aldehyde dehydrogenase-I	Decreased	Decreased	[87]
Carbonic anhydrase-III	Decreased	Decreased	[88]
Carbonyl reductase	Decreased	Decreased	[88]
c-*myc*	Increased	N.D.	[12]
Surfactant protein A	Increased	N.D.	[12]

*N.D., not determined.

TABLE IV. Summary of Frequent Allelic Loss in Hybrid Mouse Lung Carcinomas*

Chromosome	Identified target gene	Frequency (%)	Strain	Reference
1	None	5/36 (14)	$AC3F_1$	[57]
		5/15 (33)	CDF_1	[57]
4	p16	6/16 (38)	CDF_1	[10]
		18/36 (50)	$AC3F_1$	[58]
		23/45 (51)	$C3AF_1$	[10, 58]
		41/102 (40)	$B6C3F_1$	[56]
		12/24 (50)	$AC3F_1$	[56]
		11/24 (46)	$C3AF_1$	[56]
11	p53	6/8 (75)	$B6C3F_1$	[55]
		4/36 (11)	$AC3F_1$	[57]
		19/72 (26)	$C3AF_1$	[57]
12	None	10/36 (28)	$AC3F_1$	[57]
14	Rb	1/8 (12)	$B6C3F_1$	[55]
		10/36 (28)	$AC3F_1$	[57]
		8/27 (29)	$C3AF_1$	[57]
		4/15 (27)	CDF_1	[57]

*$AC3F_1$, (A/J × C3H/HeJ)F_1; CDF_1, (BALB/cJ × DBA/2J)F_1; $C3AF_1$, (C3H/HeJ × A/J)F_1; $B6C3F_1$, (C57BL/6J × C3H/HeJ)F_1.

nate reading frame; p16 and p19ARF have different first exons, referred to as exon 1α and exon 1β, respectively. The polymorphism resulting in a substitution at position 51 of p16 also produces a substitution at codon 72 of p19ARF. The two variants of p19ARF encode histidine or arginine at codon 72 with arginine co-segregating with H18 and V51. The above-mentioned LOH in lung carcinomas of intervariant F1 hybrids showed significant bias for loss of the allelic form coding for a H18 and V51, regardless of donor parent gender. For example, this allele was lost in 100% of (BALB/c x DBA/2J) F1, 84% of (C3H/HeJ x A/J) F1, 82% of (C57BL/6J x C3H/HeJ) F1, and 72% of the (A/J x C3H/HeJ) F1 lung tumors (biased allele lost is underlined). Studies using immunohistochemical analysis found that p16 inactivation is a frequent genetic defect in lung tumor progression [65] (Liu et al., unpublished data). Markedly reduced or absent p16 protein were affected in approximately half of the A/J and (C3H/HeJ x A/J) F1 lung adenocarcinomas, some of which revealed only focal areas of loss [65] (Liu et al., unpublished data). Many areas of loss were subsequently microdissected and shown by multiplex PCR analysis to display deletions of the p16 gene. Whereas hemizygous loss of the A/J allele was observed in 80% of the (C3H/HeJ x A/J) F1 tumors with no detectable p16 protein, 40% of the A/J tumors that showed an absence of p16 protein exhibited homozygous loss of the p16 gene. These results are consistent with the notion that the A/J allelic variant of p16 and/or p19ARF is a more potent growth/tumor suppressor. Our experiments have shown that transcriptional levels of the two mouse variants of the p16 and/or p19ARF do not differ significantly (Herzog et al., unpublished data). These data may suggest, therefore, that the C3H/HeJ variant of p16 and/or p19ARF exerts little selective pressure for its allelic loss in tumorigenesis. This possibility is currently being investigated.

A second region of LOH on mouse chromosome 4 was localized to distal microsatellite markers D4MIT54 and D4MIT158, syntenic to human chromosome 1p36, which is also commonly affected by LOH in multiple cancer types [58,66,67]. This region (about 3 cM) was affected by loss of heterozygosity in 44% of the lung adenocarcinomas tested. Either of the two loci on chromosome 4 underwent allelic loss in only 2% of the lung adenomas from (A/J x C57BL/6J)F_1, (C3H/HeJ x A/J) F_1, and (A/J x C3H/HeJ) F_1 mice [10,58]. These results suggested a role for the resident tumor suppressor loci in the progression or malignant conversion of mouse lung tumors.

Similarly, a pattern of LOH on chromosome 14 indicated that more than one tumor suppressor locus may reside on this chromosome [57]. Twenty-eight percent of the hybrid mouse lung adenocarcinomas displayed loss of heterozygosity on this chromosome. The Rb gene resides on chromosome 14 and has been an implied target for the LOH observed. However, a second region distantly centromeric to the Rb gene was found to undergo LOH at a frequency slightly greater than that observed for Rb [57]. This region of chromosome 14 has homology with the human chromosome 3p21–24, which is suspected to harbor a tumor suppressor gene based on frequent allelic loss in lung tumors [68].

That Rb inactivation plays a role in lung tumorigenesis has also been suggested by results showing a reduction in Rb expression in mouse lung adenomas and adenocarcinomas [12, 65] (Liu et al., unpublished results). For example, Rb mRNA expression was reduced 6–10-fold in all 25 of the (A/J x C3H/HeJ) F_1, A/J, and BALB/cJ lung adenomas examined in one study without detectable loss of heterozygos-

ity (Table III) [12]. Similarly, reduced Rb protein levels were observed in a high percentage of (C3H/HeJ x A/J) F_1 lung adenocarcinomas and A/J lung adenomas and adenocarcinomas using immunohistochemistry [65] (Liu et al., unpublished data). Interestingly, some of the adenocarcinomas that displayed Rb loss also showed focal loss of p16. In contrast to these findings, Western blot analysis of A/J adenocarcinomas showed little variation from the normal level of pRb expression, but significant variation between tumors in p16 protein levels [69]. Northern analysis also has shown somewhat higher levels of p16 mRNA in A/J lung adenomas and adenocarcinomas in comparison to normal surrounding tissue [65]. These analyses, therefore, may not be suited to detect focal aberrations. These results suggest that loss of Rb function may play a role early in mouse lung tumorigenesis, and that Rb and p16 inactivation occur together in lung adenocarcinomas. This observation is inconsistent with what has been reported in several human cancer types [70], including those of the lung, where this G1-S phase regulatory pathway is seen to function as a unit, with the alteration of one component precluding the selection for alterations in the remaining constituents [46]. However, unlike its human counterpart, mouse p16 appeared not to be influenced by Rb status in cultured fibroblasts [71]. A plausible scheme in mouse lung tumorigenesis may be that Rb down regulation occurs in lung adenomas and persists into malignancy, while loss of p16 occurs predominantly in adenocarcinomas, suggesting temporal specificity for inactivation of both in these tumors.

Recently, p53 germline mutations were examined in mouse lung susceptibility to methylnitrosourea [72]. p53 transgenic mice with a germline missense mutation (Ala135Val) were crossbred with A/J mice to study function of the p53 gene in mouse lung carcinogenesis. An average of 22 lung adenomas were observed in p53+/- mice and an average of 7 tumors in p53+/+ mice 16 weeks after exposure to methylnitrosourea, representing a 3-fold increase. However, this significant difference in tumor incidence was not seen when A/J mice were crossed with p53 knockout mice. These observations suggest that the mutant p53 allele increased lung tumor susceptibility.

CHEMOPREVENTION STUDIES EMPLOYING THE MOUSE LUNG TUMOR MODEL

A growing understanding of the specific molecular changes during lung tumor progression makes it possible to elucidate the molecular mechanism(s) of lung cancer chemoprevention by certain agents, develop surrogate endpoint biomarkers for use in clinical lung cancer chemoprevention trials, and potentially develop new and effective chemopreventive agents by targeting key genetic changes detected during lung tumorigenesis. The lung tumor model has been used extensively for chemoprevention studies [72]. For example, phenethyl isothiocyanate was found to be an effective chemopreventive agent against 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)-induced A/J mouse lung carcinogenesis [73]. As do most cancers, lung cancer develops over an extended period, apparently progressing sequentially from hyperplasia → dysplasia → adenoma → carcinoma → invasive cancer. The mouse lung tumor model progresses through many of these same stages. However, most chemopreventive studies in mice have employed development of adenomas as endpoints.

Wattenberg discussed two primary classes of chemopreventive agents based on their mechanisms of action—blocking agents and suppressing agents [75]. Most known carcinogens (e.g., aflatoxins, aromatic amines, nitrosamines, polycyclic hydrocarbons) need to be activated in the host to mutagenic and carcinogenic metabolites. Various mechanisms might effectively "block" the initial steps in carcinogenesis, e.g., altering the activities of various phase I enzymes (mostly cytochrome P450s) or phase II enzymes (conjugating enzymes), either altering production of mutagenic or carcinogenic moieties or increasing inactivation of genotoxic or carcinogenic moieties, directly blocking enzymes which activate the procarcinogens, or directly binding to or inactivating active carcinogenic or mutagenic moieties.

Most studies (see Table V) that identify chemopreventive agents in the mouse lung adenoma model have focused on the effects of these blocking agents. Several studies have reported on the inhibitory effects of isocyanates [72–75]. Other agents that block carcinogenic activation in the mouse lung adenoma assay include 5,6 benzoflavone, 2(3)-*tert*-butyl-hydroxyanisole (BHA), ethoxyquin, diallyl sulfide,

and sulindac (Table V) [76–79]. However, problems are associated with the typical chemopreventive studies employed to identify blocking agents in this model system. Most of these blockers show a marked preference for a particular class of carcinogen. For example, phenethylisothiocyanate (PEITC) is highly effective against various nitrosamine carcinogens (e.g., NNK, diethylnitrosamine) but highly ineffective against many other classes of carcinogens, e.g., polycyclic hydrocarbons. Some investigators have proposed the use of a combination of carcinogens, e.g., NNK and benzo(*a*)pyrene, which presumably may more accurately reflect the carcinogenic effects of cigarette smoke, the agent we are attempting to model. Also, most carcinogens are administered experimentally in large bolus doses, while environmentally most carcinogens are administered repeatedly at relatively low doses. This lower repeated dosing, more typical of environmental exposure to carcinogens, may make these compounds more susceptible to manipulation by blocking agents. One example of this is the finding of Pepin and coworkers [76] that sulindac is effective against NNK administered at low doses continually in drinking water, though totally ineffective against large bolus doses of the same compound. Most carcinogens are administered systemically, i.p. or i.g., in contrast to cigarette smoke, which is initially administered locally. This may lead to problems if a given blocking agent induces phase I and phase II enzymes in the liver and colon and thereby decreases the levels of carcinogen reaching the lung. Many blocking agents, particularly those that directly block the cytochromes that activate procarcinogens, have themselves been given as bolus doses shortly before the administration of carcinogen. Thus, it will be difficult to achieve a similar set of circumstances in humans. A final problem with chemopreventive studies is that the initiating agent employed is not cigarette smoke. Even though combinations of carcinogens may closely parallel it, cigarette smoke has literally hundreds of components with carcinogenic or promoting properties, making it more difficult to determine the immediate relevance of one or two carcinogenic agents.

Another type of chemopreventive agent is the suppressing agent. This term defines agents which act following the initiation stage of carcinogenesis. Chemopreventive agents with suppressive activities in the mouse lung adenoma assay include 2-difluromethylornithine (DFMO), perillyl alcohol, chalcones, myo-inositol, dexamethasone, budesonide, tea extract, lovastatin, and farnesol (Table V) [80–84] (Lubet et al., unpublished data). Given that continual exposure to mutagenic carcinogens (cigarette smoke) may occur even after initial dysplasia is achieved, it may be much more difficult to completely differentiate between suppression and blocking during progression of the disease in smokers. Nevertheless, agent(s) that are effective when given after a bolus dose of

TABLE V. Agents That Have Chemopreventive Activity in the Mouse Lung Adenoma Assay*

Chemical	Carcinogen employed	References
5,6 benzoflavone	B(*a*)P, MCA	[77]
Ascorbic acid	B(*a*)P	[89]
b-naphthoflavone	B(*a*)P, MCA	[90, 91]
BHA	B(*a*)P, Urethane, DEN	[77]
Biochanin A	B(*a*)P	[92]
Black tea extracts	NNK, DEN	[80]
Budesonide	B(*a*)P	[93, 94]
Caffeine	B(*a*)P	[89]
Chalcones		[81]
Dexamethasone	B(*a*)P, NNK	[82]
Dexamethasone + inositol	B(*a*)P, NNK	[82]
DFMO	B(*a*)P	(Lubet et al)**
Diallyl sulfide	B(*a*)P, NNK	[78–79]
D-limonene	NNK	[95]
Ellagic acid	B(*a*)P	[96]
Ethoxyquin	B(*a*)P, DEN	[77]
Farnesol	B(*a*)P	(Lubet et al)**
Ganoderma lucidum	B(*a*)P	[87]
Green tea extracts	NNK, DEN	[80]
Indole-3-carbinol	NNK	[97]
Lovastatin	B(*a*)P	[81]
Myo-inositol	B(*a*)P, NNK	[82]
PEITC	NNK	[74]
Perillyl alcohol	NNK	[84]
PHITC	NNK	[74]
Red ginseng extract	B(*a*)P	[89]
Sodium cyanate	B(*a*)P	[98]
Soybean lecithin	B(*a*)P	[89]
Sulindac	NNK	[76]
Tannic acid	B(*a*)P	[99]

*BHA, 2(3)-*tert*-butyl-hydroxyanisole; DFMO, 2-difluromethylornithine; PEITC, phenethylisothiocyanate; PHITC, phenhexylisothiocyanate; B(*a*)P, benzo(*a*)pyrene; MCA, 3-methylcholanthrene; DEN, diethylnitrosamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
** unpublished data.

carcinogen in the mouse lung adenoma model will be considered suppressing agents. This can vary from agents that cause cellular differentiation, to agents which may specifically alter enzymatic function in initiated cells, to agents that cause cell death or inhibit further growth in initiated cells. Chemopreventives can either be agents which apparently act on relatively early preinvasive lesions or agents which may work either early or late during the process of carcinogenesis (e.g., antiestrogens in breast cancer or cyclooxygenase inhibitors in colon).

Table V also summarizes a variety of agents which have been examined for chemopreventive activity in the mouse lung adenoma assay. One would expect that these agents would be equally effective against lesions induced by virtually any carcinogen. However, most of these agents have not been tested on lesions induced by different carcinogens. Perhaps the most effective suppressing agents used to date are tea extracts, which have inhibited adenoma multiplicity more than 65% in a number of studies. Certain of the more common classes of suppressor agents have not been routinely tested in the mouse lung adenoma model (e.g., vitamin D analogs, differentiating agents such as sodium butyrate, etc.). Nevertheless, a significant list of at least partially effective agents has been generated. This list is not meant to be inclusive but rather to give examples of agents which have been tested and may warrant further testing. Many chemopreventive agents which have shown efficacy in a variety of carcinogenesis models of other organs—e.g., DFMO (a specific inhibitor of ornithine decarboxylase), dexamethasone (glucocorticoid, anti-inflammatory), budesonide (a synthetic glucocorticoid), myo-inositol, green tea and black tea extracts—demonstrated significant efficacy in the mouse lung adenoma model. It may well prove that some combination of these agents will be particularly effective in the mouse lung tumor model.

FUTURE PROSPECTS

Future avenues for exploration employing the mouse lung adenoma model should include combinations of chemopreventive agents, which may be significantly more effective than any one agent alone, e.g., myo-inositol + dexamethasone. Pulmonary administration of chemopreventive agents should be explored. Administration of certain agents by aerosol may decrease systemic toxicity and perhaps even increase efficacy. Preliminary studies have shown that PEITC and the glucocorticoid budesonide are highly effective when administered by this method. Agents for specific genetic lesions should be developed. As discussed in this article, perhaps the most common genetic alteration observed in mouse lung tumors is mutation in the K-*ras* gene. Since this gene must be isoprenylated to be active, chemicals that specifically block this reaction may show activity against development of these tumors. Part of the activity of both lovastatin and farnesol (Table V) is likely related to their ability to alter isoprenylation. Preliminary studies employing highly specific inhibitors of farnesyltransferase or geranylgeranyltransferase appear to exhibit activity in this specific tumor model. Although we have briefly discussed protocols related to K-*ras*, other possible gene targets (RB, p16, etc.) are frequently altered and may be useful for chemoprevention. The mouse lung adenoma assay, with its routine alterations in certain oncogenes and tumor suppressor genes, may prove particularly applicable to studies in antisense gene therapy. Development of a cigarette smoke–induced mouse model of lung tumorigenesis would appear to be of great potential use, particularly when looking for agents that block tumorigenesis. Further identification of mouse lung susceptibility genes and characterization of transgenic models of human cancer genes will provide more appropriate animal models for familial lung cancer in humans. And finally, a systematic analysis of genetic alterations and differentially expressed genes in mouse lung tumors treated with and without known chemopreventive agents will contribute significantly to the development of surrogate endpoint biomarkers for economical and efficient clinical testing of prospective chemopreventive agents.

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PRECLINICAL AND CLINICAL MODELS OF LUNG CANCER CHEMOPREVENTION

Ming You, MD, PhD, and Gerald Bergman, PhD

Lung cancer is the leading cause of cancer deaths in men and women in the western countries, including North America.[5] Epidemiologic and laboratory animal model studies have demonstrated that smoking and environmental exposure to carcinogens are closely linked to increased lung cancer risk.[16, 24, 101, 102] Tobacco exposure has been implicated in 90% of lung carcinomas, and smokers have a 20-fold greater risk of developing lung cancer when compared with persons who have never smoked.[79] As many as 50% of all lung cancer cases occur in former smokers and lung cancer is positively correlated with pack-years smoked.[13, 58] The percentage of the US adult population that smokes peaked at almost 50% in the late 1960s, and is currently about 26%.[64] Although about half of all people who have ever smoked are now former smokers, the others are unable or are unwilling to stop smoking. For these reasons, a potentially important approach to reducing the large number of tobacco-caused cancer deaths is chemoprevention.

Chemoprevention is the use of pharmacologic or natural agents to inhibit the development of cancer. A primary mode of chemoprevention action includes reversing the progression of premalignant cells by stimulation of the cell to repair DNA or other cell damage that initiates

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carcinogenesis. Numerous studies[29] have found that chemoprevention methods can prevent or improve the outcome of a wide variety of cancers. This approach is especially useful in targeting persons who are at high risk for developing cancer, such as patients who have a genetic predisposition to cancer, or patients who are at high risk of developing secondary primary tumors after surgical removal of a tumor.[29] The targets for pharmacologic intervention are the various stages of preneoplastic development including hyperplasia and dysplasia.

There are two major classes of cancer chemopreventive agents: blocking agents and suppressing agents.[35] *Blocking agents* prevent metabolic activation of carcinogens to reduce the likelihood of DNA damage. *Suppressing agents* block expansion of carcinogen-initiated cells by suppressing cell replication or by causing apoptosis of precancerous or cancerous cells. Promising work also has been completed on reversing abnormal differentiation with hormone-like agents and various other chemopreventive agents that are largely noncytotoxic.[34]

Smoking cessation and consumption of fruits and vegetables containing chemopreventive agents are associated with reduced risk for development of lung cancer according to several epidemiologic studies.[100] Screening of potential chemopreventive agents using animal lung models has revealed that many chemicals possess inhibitory effects and may reduce the risk of human lung cancer.[24] Therefore, identification and evaluation of minor constituents of foods, their synthetic analogues, and other agents that have chemopreventive properties should produce new chemoprevention strategies for lung cancer. This review provides an overview of recent progress in the development of chemopreventive agents against human lung cancer.

MOLECULAR CARCINOGENESIS OF LUNG CANCER

Cancer development is progressive, involving increasing genetic mutations with time, which results in progressively higher levels of cell abnormalities. Similar to other types of cancer, lung cancer develops as a multi-stage process involving the accumulation of genetic alterations that affect several proto-oncogenes and tumor suppressor genes.[24, 58] As illustrated in Figure 1, many of the known genetic changes are common to both human and mouse lung tumors. Use of chemoprevention is most effective in the early stages of this progression, preferably before moderate dysplasia. Thus, a better understanding of the lung carcinogenesis process is critical not only for the identification of chemopreventive agents that can inhibit the various stages of carcinogenesis but also for the rational development of intermediate biomarkers useful in clinical chemoprevention trials.

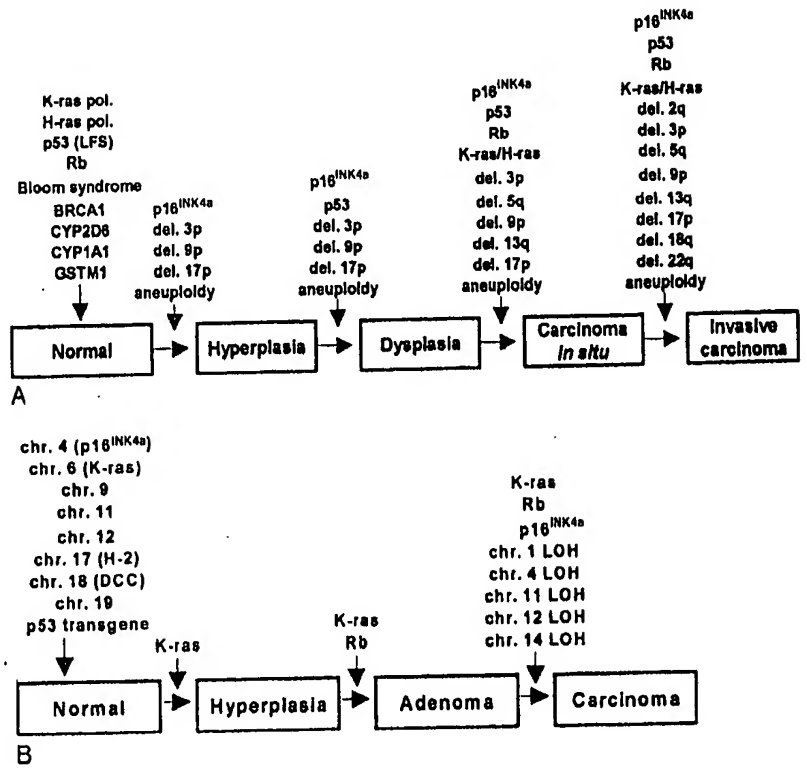

Figure 1. Genetic alterations in lung carcinogenesis. Many of the known genetic changes are common to both human *(A)* and mouse *(B)* lung tumors. Use of chemoprevention is most effective in the early stages of progression before hyperplasia. *(Adapted from* Herzog CR, Lubet RA, You M: Genetic epigenetic alterations in mouse lung tumors: Implications for cancer chemoprevention. J Cell Biochem 28/29S:49–63, 1997; with permission.)

Genetic Alterations in Human Lung Cancer

Evidence from studies of familial aggregation of lung cancer suggests that genetic factors are involved in human lung tumor development.[20, 49, 55, 74, 76, 86] Specifically, segregation analysis of lung cancer proband families indicates that a Mendelian codominant inheritance of a rare major autosomal gene is involved.[73] This gene has been shown to account for 69%, 47%, and 22% of the cumulative incidence of lung cancer in patients at ages 50, 60, and 70, respectively.[73] The identification of this gene currently is being pursued by several groups. In addition to the gene at this locus, several other genetic factors have been linked to lung cancer susceptibility. For example, a K-ras intron polymorphism and the tandem repeats in the H-ras 3′-untranslated region have been found to correlate with an increased lung cancer incidence.[53, 83] Lung cancer also appears at an increased rate in several genetic syndromes

including the Li-Fraumeni syndrome (LFS), hereditary retinoblastoma, familial breast cancer (BRCA1), and the Bloom syndrome.[19, 33, 44, 72, 82] Finally, genetic differences in the genes (CYP2D6, CYP1A1, and GSTM1) responsible for metabolism of tobacco carcinogens also have been implicated in susceptibility to lung cancer.[10, 77, 85] Genetically predisposed individuals can be used as high-risk populations both for chemoprevention trials and for developing of effective chemoprevention strategies using susceptibility genes as targets.

Molecular changes in proto-oncogenes and tumor suppressor genes have been dectected in all stages of lung carcinogenesis. Several genetic changes, including hypermethylation of p16, p53 mutation, and deletion of 3p, 9p, and 17p, have been detected frequently in hyperplastic and dysplastic lesions of the lung.[7, 38, 57, 67, 81] These changes together with aneuploidy are considered primary candidates for intermediate biomarkers in clinical trials, because patients with lung hyperplasia and dysplasia are the main source of cohorts for lung cancer chemoprevention studies. Activation of the K-ras gene is observed frequently in lung adenocarcinomas in smokers.[70] Inactivation of p16 by hypermethylation and homozygous deletion has been detected in the majority of non–small cell lung carcinomas.[57, 67] Furthermore, p53 mutations and Rb gene inactivation have been seen frequently in invasive carcinomas.[12, 103] Loss of heterozygosity of five loci (3p, 5q, 9p, 13q, and 17p) also has been frequently observed in carcinomas in situ of the lung.[18] Deletion of 18q and 22q is seen only in invasive carcinomas, suggesting that the genes in these two loci may be responsible for malignant progression of lung cancer.[78]

These genetic alterations found in precancerous and cancerous lesions of the lung are the primary candidates for use as intermediate endpoint biomarkers in clinical chemoprevention trials of lung cancer. Development of appropriate intermediate endpoint biomarkers becomes critical for efficiently and economically conducting clinical chemoprevention trials. Other intermediate biomarkers for lung cancer include histopathology, sputum cytology, computer-assisted image analysis, and molecular biomarkers.[8] Recently, a laser-induced fluorescent emission (LIFE) bronchoscope has been used to identify early histologic changes of precancerous lesions precisely.[39] In clinical trials that evaluated the efficacy of retinoids, the expression of retinoic acid receptors was found to be a valuable biomarker.[48] Finally, the recently proposed field cancerization concept could be very useful in monitoring genetic alterations of multiple genes.[17] Additional intermediate biomarkers are being developed through systematic analysis of gene expression and genetic alterations in precancerous lesions.

Genetic Alterations in Mouse Lung Tumors

As shown in Figure 1, similar molecular changes seen in human lung cancer also have been observed in mouse lung tumors. Susceptibility to chemical induction of lung tumors in mice varies according to the strain.[51] Genetic linkage analyses using various mouse crosses have revealed a series of pulmonary adenoma susceptibility (Pas) genes, such as Pas1 (chromosome 1), Pas2 (chromosome 17), and Pas3 (chromosome 19), and pulmonary adenoma resistant (Par) genes, such as Par1 (chromosome 11), Par2 (chromosome 18), Par3 (chromosome 4), and Par4 (chromosome 12).[24] The K-ras proto-oncogene is a candidate for Pas1, and the p16 gene is a candidate for Par3.[23, 46] Recently, p53 transgenic mice with a germline mis-sense mutation were found to have a significantly increased susceptibility to chemically induced lung tumorigenesis, making it a lung tumor model for LFS in humans.[47] The K-ras proto-oncogene also is frequently activated in both mouse lung adenomas and adenocarcinomas.[105] Loss of heterozygosity of chromosomes 1, 4, 11, 12, and 14 was seen frequently in lung adenocarcinomas, suggesting that these changes may contribute to the progression of mouse lung carcinogenesis.[25] Alterations of known tumor suppressors, such as p16, Rb, and p53, also have been detected in lung tumors.[24] In general, genetic changes found in mouse lung tumors have remarkable similarities to those existing in humans.

PRECLINICAL EFFICACY OF CHEMOPREVENTIVE AGENTS IN RODENT LUNG CARCINOGENESIS MODELS

Because the histopathologic changes, stages of tumor progression, and molecular changes in mouse lung adenocarcinomas are similar to those in human lung adenocarcinomas, the mouse lung tumor model has been used extensively to evaluate the efficacy of putative lung cancer chemopreventive agents.[24, 50] Among the more than 50 different agents tested, so far four groups of chemicals have shown significant efficacy against mouse lung tumor development: glucocorticoids, isothiocyanates, tea polyphenols, and nonsteroidal anti-inflammatory drugs (NSAIDs).

Glucocorticoids

Glucocorticoids were found to be strong inhibitors of carcinogenesis in skin, forestomach, and lung in rodents.[6, 15, 88, 95] Wattenberg et al[95] reported that dexamethasone, a synthetic glucocorticoid, inhibits lung tumorigenesis by 56%, presumably by promoting the maturation of type II alveolar cells, a major target of lung carcinogens. An 86% inhibition of lung tumor development was observed when dexamethasone was given together with myo-inositol.[95] Similarly, another synthetic glucocorticoid, budesonide, was found to inhibit benz(a)pyrene-induced lung

tumorigenesis by 89%[96]; however, the use of glucocorticoids as chemopreventives currently is not feasible owing to the development of systemic toxic effects. To minimize the systemic toxic effects, glucocorticoids such as budesonide can be delivered successfully by aerosol, and, by this method, they inhibit lung tumor development by over 90%.[99]

Isothiocyanates[32, 59, 60, 61, 75]

The representative compound from this group of agents is the phenethyl isothiocyanate (PEITC).[59, 75] PEITC, common in many cruciferous vegetables, has shown specific inhibitory effects of rodent tumor development induced by tobacco-specific nitrosomes such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) when administered during the initiation stage. In a rodent lung model, PEITC is capable of complete abolition of NNK-induced carcinogenesis,[59] but it is not effective against some other classes of chemical carcinogens. The basis for the chemopreventive effects is largely due to its inhibition of specific cytochrome P450 enzymes. More recently, administration of PEITC by inhalation led to a strong inhibitory effect against NNK-induced mouse lung tumor development.[75] This approach may decrease systemic toxicity and increase the efficacy of the test agent. Other isothiocyanates also have been shown strong inhibitory effects against NNK-induced carcinogenesis in mouse lung tumor models.[32, 60, 61]

Tea Polyphenols[89, 104]

Green tea is a potent inhibitor of carcinogenesis in skin, lung, forestomach, esophagus, liver, colon, and mammary glands in rodent models. Green tea contains flavanols or catechins such as (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3-gallate (EGCG), (+)-gallocatechin, and (+)-catechin. These polyphenols have various biologic activities including antioxidation, modulation of enzyme systems for metabolizing chemical carcinogens, inhibition of nitrosation reactions, scavenging of activated metabolites of chemical carcinogens, and inhibition of tumor promotion. Green tea and one of its components, EGCG, have been shown to inhibit NNK-induced mouse lung tumorigenesis by 63% and 28%, respectively.[89, 104] Green tea also was found to inhibit the growth or even cause the regression of established benign tumors, suggesting that it may be preventive against all stages of carcinogenesis.[90]

NSAIDs[14, 31, 52]

NSAIDs are strong inhibitors of carcinogenesis of tongue, esophagus, mammary gland, pancreas, uterine cervix, bladder, and lung in rodents. Castonguay et al[14, 31] reported that aspirin, acetylsalicylic acid, sulindac, ibuprofen, and piroxicam reduced NNK-induced lung tumorigenesis by 62%, 60%, 58%, 38%, and 32% in A/J mice, respectively. More recently, sulindac sulfone, a sulfone derivative of sulindac, was found to be a potent inhibitor of lung tumorigenesis in mice, reducing tumor multiplicity by about 90%.[52] Most NSAIDs showed strong efficacy against mouse lung carcinogenesis only when the carcinogen (eg, NNK) was given repeatedly at a relatively low dose rather than administered in a large bolus dose.

Other Agents

Farnesyltransferase inhibitors recently have been characterized as potential chemopreventive agents against mouse lung tumors; they include perillyl alcohol,[10] D-limonene,[94] and lovastatin.[22] As summarized in Table 1, many other chemopreventive agents inhibit mouse lung tumorigenesis.[1, 2, 4, 9, 21, 28, 42, 43, 45, 62, 91–93, 97, 98, 106, 107] Additional agents currently being tested by the Chemoprevention Branch of the National Cancer Institute are likely to be added to this list in the future.

Although epidemiologic evidence exists that consumption of fruits and vegetables containing compounds such as β-carotene may reduce lung cancer risk, β-carotene in combination with retinol did not protect lung tissues from NNK-induced carcinogenesis in A/J mice.[11] This result is consistent with several clinical trials conducted later using the same combination of agents in humans, in which an increased incidence in lung cancer was observed in smokers. Other chemicals that were ineffective against mouse lung tumor development include 9-cis-retinoic acid, 4-N-(hydroxyphenyl)retinamide (4-HPR), and oltipraz[63] (Lubet et al, unpublished data).

CLINICAL CHEMOPREVENTION TRIALS OF LUNG CANCER (TABLE 2)

The success in using retinoids in chemoprevention of head and neck cancers has provided insight into the specific types of cancer that was effectively prevented by retinoids.[68] A randomized, placebo-controlled study of squamous cell carcinoma of the head and neck revealed that isotretinoin has the potential to block second primary tumor development.[30] These results were considered important because second primary tumors are a major cause of death in early-stage squamous cell carci-

Table 1. CHEMOPREVENTIVE AGENTS TESTED IN RODENT LUNG TUMOR MODEL

Class of Agents	Agent	Route of Administration	Carcinogen Used	Inhibitory Effect (%)	References
Glucocorticoids	dexamethasone	diet	B(a)P	56	96
	dexamethasone + myo-inositol	diet	B(a)P	86	96
	budesonide	diet	B(a)P	89	97
	budesonide	aerosol	B(a)P	90	99
Isothiocyanates	phenethyl isothiocyanate	i.g.	NNK	97	59
	phenethyl isothiocyanate	aerosol	NNK	75	75
	3-phenylpropyl isothiocyanate	i.g.	NNK	95	60
	4-phenylbutyl isothiocyanate	i.g.	NNK	95	60
	5-phenylpentyl isothiocyanate	i.g.	NNK	96	61
	6-phenylhexyl isothiocyanate	i.g.	NNK	98	61
	8-phenyloctyl isothiocyanate	i.g.	NNK	68	32
	10-phenyldecyl isothiocyanate	i.g.	NNK	73	32
	1,2-diphenylethyl isothiocyanate	i.g.	NNK	95	32
	2,2-diphenylethyl isothiocyanate	i.g.	NNK	94	32
	hexyl isothiocyanate	i.g.	NNK	85	32
	2-hexyl isothiocyanate	i.g.	NNK	97	32
	dodecyl isothiocyanate	i.g.	NNK	97	32
Tea	green tea	drinking water	NNK	85	89
	black tea	drinking water	NNK	63	89
	epigallocatechin-3-gallate	drinking water	NNK	28	104
Nonsteroidal anti-inflammatory drugs	aspirin	diet	NNK	62	14
	acetylsalicylic acid	diet	NNK	60	14
	sulindac	diet	NNK	52	14
	ibuprofen	diet	NNK	38	31
	piroxicam	diet	NNK	30	31
	sulindac sulfone	diet	NNK	90	52
Farnesyltransferase inhibitors	perillyl alcohol	i.p.	NNK	58	40
	D-limonene	i.g.	NNK	78	94
	lovastatin	diet	NNK	48	22
	farnesol	diet	NNK	35	Lubet et al*
Other compounds	ascorbic acid	drinking water	B(a)P	35	107
	α-naphthoflavone	i.p.	MCA	50	2,91,98
	butylated hydroxyanisole	i.g.	B(a)P	70	93
	biochanin A	i.g.	B(a)P	>50	42
	chalcones	diet	B(a)P	29	95
	2-difluoromethylornithine	diet	NNK	35	Lubet et al*
	diallyl sulfide	i.g.	NNK	92	28
	ellagic acid	i.p.	B(a)P, NNK	50	9,43
	2-ethynylnaphthalene	i.g.	NNK	77	1
	ethoxyquin	diet	DHPN	42	21
	ganoderma lucidum	diet	B(a)P	79	107
	7-hydroxy-1-phenyl-1-octanone	i.g.	NNK	48	45
	4-hydroxy-1-phenyl-1-pentanone	i.g.	NNK	49	45
	indole-3-carbinol	i.g.	NNK	40	62
	4-phenyl-1-butyne	i.g.	NNK	74	1
	1,4-phenylenebis(methylene)selenocyanate	i.g.	NNK	78	62
	5-phenyl-1-pentyne	i.g.	NNK	95	1
	red ginseng extract	drinking water	B(a)P	44	106
	sodium cyanate	i.g.	B(a)P	46	92
	soybean lecithin	drinking water	B(a)P	61	107
	tannic acid	diet	B(a)P	48	4

i.g. = intragastric gavage; i.p. = intraperitoneal injection; B(a)P = benzo(a)pyrene; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MCA = 3-methylcholanthrene; DHPN = N-bis(2-hydroxypropyl)nitrosamine; * = unpublished data.

Table 2. CLINICAL CHEMOPREVENTION TRIALS OF LUNG CANCER

Compound	Dose/day	Cohort	No. Patients	Study Length	Endpoint	Results	References
13-cis-retinoic acid	1–2.5 mg/kg	patients with sputum atypia	26	4–40 weeks	sputum cytology	no effect	71
13-cis-retinoic acid	1 mg/kg	smokers with dysplasia or squamous metaplasia	86	6 months	squamous metaplasia	no effect	41
13-cis-retinoic acid	30 mg/day	patients with resected stage I NSCLC (Intergroup study)	1470	NA	second primaries	ongoing	36
13-cis-retinoic acid	NA	smokers	70	NA	intermediate biomarkers	ongoing	Hawk et al*
etretinate	25 mg	patients with sputum atypia	150	6 months	sputum cytology	no effect	3
etretinate	25 mg	smokers with squamous metaplasia	101	6 months	squamous metaplasia	reduction	54
vitamin A	300,000 IU	patients with resected stage I NSCLC	307	1 year	second primaries	reduction	69
vitamin A	NA	smokers with dysplasia	80	NA	dysplasia	ongoing	Hawk et al*
β-carotene	20 mg	smokers	114	14 weeks	micronuclei in sputum	reduction	87
β-carotene	50 mg/2 days	smokers and nonsmokers	22,071	12 yrs	lung cancer incidence	no effect	27
4-HPR	NA	smokers with dysplasia or squamous metaplasia	123	NA	squamous metaplasia	ongoing	Hawk et al*
oltipraz	NA	smokers with resected lung cancer	104	NA	second primaries	ongoing	Hawk et al*
oltipraz	NA	smokers	36	NA	toxicity	ongoing	Hawk et al*
PETTC	NA	smokers with resected lung cancer	25	NA	toxicity	ongoing	Hawk et al*
anethole trithione	NA	smokers with dysplasia	90	NA	dysplasia	ongoing	Hawk et al*
Vitamin B12, folic acid	0.5 mg, 10 mg	smokers with sputum atypia	73	4 months	sputum cytology	not conclusive	26
β-carotene, vitamin E	20 mg, 50 mg	smokers	29,133	5–8 yrs	lung cancer incidence	18% increase	84
β-carotene, vitamin A	30 mg, 25,000 IU	smokers	18,314	4 yrs	lung cancer incidence	23% increase	65, 66
β-carotene, retinol	50 mg, 25,000 IU	former asbestos workers	755	3–5 years	sputum cytology	no effect	56
13-cis-retinoic acid, vitamin E, 9-cis-retinoic acid	1 mg/day, 1200 IU/day, 50 mg/day	patients with resected stage I NSCLC	225	NA	squamous metaplasia	ongoing	36
N-acetylcysteine	600 mg	(Eurocan study)	2600	2 years	second primaries	ongoing	80
aspirin, vitamin E	NA	female nurses	62,600	NA	lung cancer incidence	ongoing	Hawk et al*
β-carotene, vitamin C	NA	smokers	300	NA	intermediate biomarkers	ongoing	Hawk et al*
α-interferon, 13-cis-retinoic acid, vitamin E	NA	smokers with dysplasia or squamous metaplasia	35	NA	squamous metaplasia	ongoing	Hawk et al*

NSCLC = non-small cell lung cancer; 4-HPR = 4-N-(hydroxyphenyl)retinamide; PETTC = phenethyl isothiocyanate; NA = not available; * = unpublished data.

noma. Success of this and similar studies has led to an investigation of retinoids as chemopreventives in certain lung cancers. Clinical trials in smokers using retinoids have generated largely negative and mixed results[3, 37, 41, 54, 69, 71, 80] (Hawk et al, unpublished data).

The selection of β-carotene as a potential chemopreventive for the lung was based largely upon epidemiological data that showed a positive correlation between the consumption of β-carotene-rich foods, and high blood levels of β-carotene and reduced lung cancer risk. Several large chemoprevention trials using β-carotene, retinoids, or vitamin E were initiated subsequently, but none of them showed any chemopreventive effects.[27, 36, 65, 66, 84] The complex relationship between chemoprevention and smoking was illustrated in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) completed in Finland[84] and in the Beta-Carotene and Retinol Efficacy Trial (CARET) in the United States.[65, 66] The results showed that these supplements alone or in combination with retinol *increased* lung cancer risks in smokers.

ATBC Trial[84]

The Finnish α-tocopherol/β-carotene cancer prevention study randomly separated 29,133 male smokers into four groups. One used the daily supplements of either 20 mg of β-carotene or 50 mg of α-tocopherol, or both, or a placebo, for an average of 6 years. Of the 876 new cases of lung cancer reported, the incidence of lung cancer was 18% higher in men who took β-carotene supplements compared with men who did not. The total mortality was also 8% higher ($P = 0.02$) among those who took β-carotene supplements. No difference in cancer incidence or overall mortality was found between the group that took vitamin E and those who did not.

CARET Trial[65, 66]

The β-carotene and retinol efficacy trial involved more than 18,000 men and women, all of whom were at high risk of lung cancer due to either smoking or asbestos exposure. The experimental group received 30 mg of β-carotene and 25,000 IU of retinol per day. The trial was terminated 20 months early because the results appeared to be similar to those seen in the Finnish study.[66] Fully 338 new cases of lung cancer were in the experimental group, and the deleterious effects of the supplements appeared to be greater among the heavy smokers who continued to smoke during the trial than among both those who quit smoking before the trial and nonsmokers. These results support the perception

that the key factor is not simply smoking but primarily *current* smoking behavior. Because the study was terminated early, the results are difficult to interpret, but they do indicate that the supplements used adversely affected current smokers.

Physicians' Health Study[27]

The Physicians' Health Study involved about 22,000 American physicians who were randomized into two populations. The first received 50 mg of β-carotene on alternate days, and the second group received a placebo. In this study, only 11% were current smokers and 39% were former smokers. During the 12-year study, 170 new cases of lung cancer were diagnosed. Eighty-two new cases of lung cancer occurred in the group receiving β-carotene, and 88 in the placebo group, a difference that was not significant. A major difference between the physicians and the Finnish populations was smoking behavior.

The general conclusion of these studies is that no evidence that β-carotene has chemopreventive effects against lung cancer, and they appear to adversely affect current smokers. Plausible reasons for these findings include differences in the combination of β-carotene and numerous other vitamins, including especially vitamins A, C, and E. The protocol and the dosage used in the trial may have elicited toxic effects. Such toxicity may be synergistic with tobacco smoke in promoting human lung cancer. Furthermore, this synergy appears to be dose-dependent. For example, the Physicians' Health Study used considerably lower doses of β-carotene than did both the ATBC and CARET studies and it found less effect. If this scenario is correct, it may explain why many chemopreventives are ineffective in preventing lung cancers in smokers.

SUMMARY

In smokers, β-carotene, retinol, and vitamins E and C appear to have little or a negative effect against human lung cancer development. Similarly, these chemicals have generally failed to inhibit lung tumorigenesis in rodents. The agents that have been shown to inhibit lung tumorigenesis in rodents, such as glucocorticoids, green tea, NSAIDs, and isothiocyanates, have not been tested yet in humans. These agents may be more effective in preventing human lung cancer in smokers than are the chemicals tested so far, especially if they are delivered by inhalation route.

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Trial of a New Medium-Term Model Using Benzo(a)pyrene Induced Lung Tumor in Newborn Mice

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Abstract. *A new medium-term in vivo model was tried using pulmonary adenoma induced by benzo(a)pyrene (BP) in newborn mice. Both inbred mice such as C57BL/5J, C57BR/cdJ. A/J mice and non inbred N:GP(S) mice were used. Benzo(a)pyrene was injected in the subscapular region of newborn mice within 24 hours after birth at a dose of 0.5 mg and 1 mg per mouse, respectively. After 9 weeks lung tumor induced in N:GP(S) and A/J mice but in the other mice. The dose showing a 50% tumor incidence was found in N:GP(S) mice to be 0.5 mg of BP but the tumor incidence was very high in A/J mice even at 40 μg of BP, the lowest dose in this experiment. To verify the utility of this model, ascorbic acid, carrot, beta carotene, soybean lecithin, spinach, Sesamum indicum, Ganoderma lucidum, caffeine, red ginseng extract, fresh ginseng and 13-cis retinoic acid, some of which are known to have anticarcinogenic activity in various animal models, were tried with this system. Ascorbic acid, soybean lecithin, Ganoderma lucidum, caffeine and red ginseng extract showed inhibition of lung tumor incidence, while fresh ginseng, carrot, beta carotene, spinach and 13-cis retinoic acid did not. This result suggested that the 9-week medium - term model using lung tumor induced by 0.5 mg of BP was useful for the screening of cancer preventive agents.*

For the detection of the carcinogenic or anticarcinogenic potential of environmental compounds various experimental systems have been developed and put into practice. Among them 2-year long-term *in vivo* carcinogenicity tests using rats, mice or hamsters have been considered to be the most reliable for the production of carcinogenic or anticarcinogenic potential in man (1, 2). However, to be internationally accepted these long-term tests must satisfy costly regulatory guidelines for appropriate facilities, long duration maintenance of animals, sufficiently large numbers of rodents and careful histopathological examination. The number of compounds which have been introduced into our environment is far beyond our capacity for such testing. For the purpose of performing mass screening of compounds, *in vitro* short-term screening assays using gene mutation, including the Salmonella/microsome assay (Ames test), chromosomal aberration and various other test systems, have been developed (3-6). Using these methods, a variety of compounds were shown to be mutagenic with apparently good correlation to their known carcinogenicity (7-11). However, as the number of compounds tested increased it became clear that the mutagenicity results did not always show a direct correlation to carcinogenicity (12-14). Recently, attention is being paid to developing an *in vivo* medium-term assay system using glutathione S-transferase placental from positive foci of rat liver (15-17). In general, the numbers of foci induced by carcinogen exposure are predictive of the numbers of neoplasms with the same phenotype that will evolve (18, 19). However, large numbers of foci can be measured as opposed to much smaller numbers of neoplasm (20) and non-carcinogens can be induced foci nonspecifically.

The mouse pulmonary carcinogenesis model is also used in carcinogenic or anticarcinogenic assays (21,22). The authors have previously shown a correlation between natural killer (NK) cell activity and lung adenoma incidence. In that experiment, NK cell activity remarkably decreased from 4 weeks of age when 1 mg urethane was administered to the mice within 24 hours after birth. The incidence of lung adenoma was 100 percent at week 9 in the urethane treated group. Considering that development of lung adenoma required a long-term latent period in the group treated with benzo(a)pyrene and also that NK cell activity normally reached a peak at week 6 and began descending at week 12 (23). Therefore, in this study, we attempted to establish a new medium-term carcinogenicity or anticarcigenicity model induced by benzo(a)pyrene in various strains of newborn mice and tested the validity of this model.

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Key Words: Benzo(a)pyrene, N: GP(S) mice, newborn, nine weeks anticarcinogenic model.

Materials and Methods

Animals. Inbred A/J, C57BL/6J, C57BR/cdJ mice were from Jackson Lab-

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oratory (USA) and N:GP(S) mice were from the National Cancer Institute (NCI, USA). Newborn mice within 24 hours after birth were used. Diet pellets were according to the NIH 7-open formula diet.

Experimental design. Difference of lung tumor incidence according to mouse strains: Newborn mice less than 24 hours old of A/J, C57BL/6J, C57BR/cdJ and N:GP(S) strains were injected subcutaneously in the scapular region with 0.02 ml of a suspension, 0.5 mg or 1 mg of benzo(a)pyrene (BP, Sigma Chemical Co., USA) in 1% aqueous gelatin, once. The carcinogen was used within 1 hour after emulsification.

Dose response effect of benzo(a)pyrene induced lung tumor in N:GP(S) or A/J mice: BP, 40, 62.5, 125, 500 or 1000 μg was injected once in A/J and N:GP(S) newborn mice.

Anticarcinogenicity of various natural products in the new model system. BP, 0.5 mg, was injected in N:GP(S) newborn mice and after weaning the following materials were administered for 6 weeks: Fresh ginseng (4 year old ginseng), water extract of red ginseng (Office of Monopoly, Korea), ascorbic acid (Sigma Chemical Co., USA) and soybean lecithin (95% phosphatides form, California Co., USA) were given in drinking water at a concentration of 12.85 mg/ml, 1 mg/ml, 10 mg/ml and 25 mg/ml, respectively. Beta-carotene (Sigma Chemical Co., USA), spinach, *Sesamum indicum*, and *Ganoderma lucidum* were ground and mixed in diet to be 500 mg/kg diet, 25% diet, 5% diet and 25% diet. Caffeine (BDH Chemicals, England) was dissolved in tap water at 1 mg or 2 mg and 1 ml was administered by oral intubation every day. 13-cis retinoic acid was kindly supplied by Hoffman-La Roche (NJ, USA) as a 10% gelatin-beadlet form and administered in the diet at a concentration of 240 mg/kg of diet. Drinking water was changed every other day and diet was prepared every other week.

Scoring of lung tumors. All mice sacrificed at the 9th week after birth. Lungs were excised and fixed in Tellyesniczk solution (100 ml of 70% ethanol, 3 ml of formalin, 5 ml of glacial acetic acid). Then the adenoma were counted by the naked eye. After counting the lungs were embedded in paraffin, then cut and stained with hematoxylin-eosin. To obtain an index of tumor incidence, the percentage of tumor bearing mice per total number of mice in each group was calculated. Tumor multiplicity was defined as the average number of tumors per mouse obtained by dividing the total number of tumors by the total number of mice per group including nontumor-bearing animals. Statistical comparisons were made using the Chi-square test for tumor incidence and Student's t test of multiplicity. A null hypothesis was rejected whenever a P value of 0.05 or less was found.

Results

The experiment for the establishment of a new anticarcinogenicity model. Histopathological analysis revealed that all lung tumors were pulmonary adenomas. Table I shows the incidence and multiplicity of lung adenoma induced by BP in various strains of mouse. Lung adenoma incidence was 46.8% and 54.4% in N:GP(S) mice at a concentration of 0.5 mg and 1 mg in BP treated groups, respectively. That of A/J mice was 86.7% and 88.3%, that of C57BL/6J mice was 1.3% and 0%, and that of C57BR/cdJ was all 0%. The dose response effect of BP in A/J and N:GP(S) mice was also examined. A single injection of 40 μg of BP, which was the lowest dose in this experiment, showed an incidence of lung tumors of 71.0% in A/J mice, which might be too high in incidence to evaluate the anticarcinogenicity of unknown compounds (Table II). However, 500 μg of BP induced 49.4% of lung tumor incidence in N:GP(S) mice (Table III).

Table I. *The incidence and multiplicity of lung tumors in four strains at the 9th week after injection of benzo(a)pyrene.*

Strains and dose	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
A/J				
1% Gelatin	M	39	2.6	0.03±0.17
	F	37	5.4	0.05±0.21
	M+F	76	3.9	0.04±0.20
Benzo(a)pyrene				
0.5 mg*	M	40	82.5	2.95±1.40
	F	35	91.4	2.71±1.48
	M+F	75	86.7	2.84±1.42
1.0 mg	M	35	85.7	2.57±1.06
	F	25	92.0	3.88±1.15
	M+F	60	88.3	3.12±1.27
C57BL/6J				
1% Gelatin	M	40	0	0
	F	40	0	0
	M+F	80	0	0
Benzo(a)pyrene				
0.5 mg	M	38	2.6	0.03±0.20
	F	40	0	0
	M+F	78	1.3	0.01±0.14
1.0 mg	M	38	0	0
	F	36	0	0
	M+F	74	0	0
C57BR/cdJ				
1% Gelatin	M	10	0	0
	F	8	0	0
	M+F	18	0	0
Benzo(a)pyrene				
0.5 mg	M	10	0	0
	F	19	0	0
	M+F	29	0	0
1.0 mg	M	26	0	0
	F	17	0	0
	M+F	43	0	0
N:GP(S)				
1%Gelatin	M	39	2.6	0.03±0.18
	F	40	2.5	0.03±0.19
	M+F	79	2.5	0.03±0.19
Benzo(a)pyrene				
0.5 mg	M	40	37.5	0.60±2.05
	F	39	56.4	0.92±1.16
	M+F	79	46.8	0.76±0.78
1.0 mg	M	33	51.5	0.79±0.56
	F	35	57.1	0.77±1.32
	M+F	68	54.4	0.78±1.02

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection

The experimental for the evaluation of the model established by BP in newborn N:GP(S) mice using various natural products. Tables IV, V, VI, VII show the incidence and multiplicity of lung tumors when they had been tested using the established

Table II. *The incidence and multiplicity of lung tumors in A/J mice at the 9th week after various doses of benzo(a)pyrene injection*

Dose	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	13	7.7	0.23±0.97
	F	18	0	0
	M+F	31	3.2	0.10±0.53
Benzo(a)pyrene 0.4 mg*	M	13	69.2	2.08±0.38
	F	18	72.2	1.94±1.20
	M+F	31	71.0	2.00±1.31
0.0625mg	M	16	93.8	2.44±0.63
	F	15	72.2	1.94±1.17
	M+F	31	71.0	2.00±0.97
0.125 mg	M	20	95.0	4.60±1.05
	F	25	92.0	3.72±1.10
	M+F	45	93.3	4.11±1.16
0.250 mg	M	19	89.5	4.74±1.63
	F	20	90.0	2.20±0.73
	M+F	39	89.7	3.44±1.78
0.5 mg	M	25	92.0	3.76±1.11
	F	20	85.0	3.25±1.37
	M+F	45	88.9	3.53±1.26
1.0 mg	M	14	92.9	5.21±1.45
	F	15	86.7	3.53±1.39
	M+F	29	89.7	4.34±1.65

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection

Table III. *The incidence and multiplicity of lung tumors in N:GP(S) at the 9th week after various doses of benzo(a)pyrene injection*

Dose	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	40	0	0
	F	40	0	0
	M+F	80	0	0
Benzo(a)pyrene 0.04 mg*	M	40	5.0	0.05±0.22
	F	40	7.5	0.08±0.28
	M+F	80	6.3	0.06±0.25
0.0625 mg	M	40	12.5	0.13±0.32
	F	40	20.0	0.23±0.33
	M+F	80	16.3	0.18±0.25
0.125 mg	M	39	38.1	0.62±0.78
	F	34	29.4	0.59±0.85
	M+F	73	34.2	0.60±0.81
0.250 mg	M	37	27.0	0.27±0.44
	F	40	27.5	0.40±0.64
	M+F	77	27.3	0.34±0.56
0.5 mg	M	40	42.5	0.73±0.85
	F	39	56.4	1.10±0.94
	M+F	79	49.4	0.89±0.91
1.0 mg	M	40	57.5	1.75±1.51
	F	40	50.0	0.83±0.83
	M+F	80	53.8	0.95±1.31

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection

Table IV. *The lung tumor incidence and multiplicity in N:GP(S) mice at the 9th week after benzo(a)pyrene injection combined with ascorbic acid, fresh ginseng or red ginseng extract.*

Groups and treatment	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	39	2.6	0.03±0.16
	F	40	2.5	0.03±0.16
	M+F	79	2.6	0.03±0.16
Benzo(a)pyrene (BP) 0.5 mg*	M	40	37.5	0.55±0.85
	F	39	56.4	0.98±1.21
	M+F	79	47.0 (100)	0.77±1.03
Ascorbic acid 10 mg/ml**	M	40	0	0
	F	40	0	0
	M+F	80	0	0
BP+ascorbic acid	M	38	23.7	0.37±0.75
	F	40	35.0	0.63±1.08
	M+F	78	29.5 (62.8)[1]	0.50±0.92
Fresh ginseng 12.85 mg/ml**	M	38	0	0
	F	39	0	0
	M+F	77	0	0
BP+fresh ginseng	M	38	36.5	0.79±1.36
	F	36	52.6	1.28±2.00
	M+F	74	44.4 (94.5)	1.04±1.68
Red ginseng extract 1 mg/ml**	M	38	2.6	0.03±0.20
	F	40	2.5	0.03±0.16
	M+F	78	2.6	0.03±0.18
BP+red ginseng extract	M	40	20.0	0.30±0.69
	F	40	35.0	0.55±0.88
	M+F	80	27.5 (58.5)[2]	0.43±0.79[1]

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection
** mg per ml in drinking water
Significantly different from the BP alone group at [1]P<0.05 and [2]P<0.01

new medium-term model in newborn N:GP(S) mice induced by 500 μg of BP. Ascorbic acid, red ginseng, soybean lecithin, *Ganoderma lucidum*, and caffeine 1 mg and 2 mg significantly inhibited the lung tumor incidence by 37.5%, 58%, 71.2%, 82.2% and 54%, respectively. The other natural products did not show any significant inhibition of lung tumor incidence.

Discussion

The present study was performed to establish a new medium-term anticarcinogenicity model induced by benzo(a)pyrene in newborn mice.

It has been reported that *in vitro* tests have been carried out in order more conveniently to identify anticarcinogenic compounds. However, their activity was often different from *in vivo* effects which appear after a relatively long term. Gamma glutamyl transpeptidase and glutathione S-transferase, which are sometimes induced by non-carcinogenic compounds and

Table V. *The lung tumor incidence and multiplicity in N:GP(S) mice at the 9th week after benzo(a)pyrene injection combined with beta carotene or soybean lecithin*

Groups and treatment	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	39	2.6	0.03±0.16
	F	40	2.5	0.03±0.16
	M+F	79	2.6	0.03±0.16
Benzo(a)pyrene (BP) 0.5 mg*	M	41	36.6	1.02±2.17
	F	39	56.4	1.38±2.08
	M+F	80	64.3 (100)	1.20±2.13
Carrot 4.4 g/day	M	40	0	0
	F	38	0	0
	M+F	78	0	0
BP+carrot	M	37	29.7	0.54±0.96
	F	39	53.8	1.15±1.36
	M+F	76	42.1 (65.5)	0.85±1.16
Beta carotene 0.5 g/g diet	M	40	0	0
	F	38	0	
	M+F	78	0	0
BP+beta carotene	M	40	37.5	0.85±1.42
	F	40	37.5	0.83±1.81
	M+F	78	37.5 (58.3)	0.84±1.62
Soybean lecithin 25 mg/ml**	M	39	0	0
	F	40	0	0
	M+F	78	0	0
BP+soybean lecithin	M	40	25.0	0.43±0.84
	F	40	32.5	0.50±0.82
	M+F	80	28.8 (44.8)[1]	0.47±0.83[2]

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection
** mg per ml in drinking water
Significantly different from the BP alone group at [1]P<0.05 and [2]P<0.001

Table VI. *The lung tumor incidence and multiplicity in N:GP(S) mice at the 9th week after benzo(a)pyrene injection combined with spinach, Sesamum indicum and Ganoderma ludidum.*

Groups and treatment	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	35	0	0
	F	34	2.9	0.03±0.17
	M+F	69	1.4	0.02±0.09
Bwnzo(a)pyrene (BP) 0.5 mg*	M	39	33.3	0.62±1.11
	F	40	37.5	0.70±1.09
	M+F	79	35.4 (100)	0.66±1.10
Spinach 25% inthe diet	M	32	0	0
	F	33	3.0	0.03±0.17
	M+F	65	1.5	0.02±0.09
BP+spinach	M	40	22.5	0.38±0.77
	F	40	22.5	0.33±0.69
	M+F	80	22.5 (63.6)	0.36±0.73[1]
Sesamum indicum 5% in the diet	M	25	0	0
	F	25	12.0	0.12±0.33
	M+F	50	6.0	0.06±0.17
BP+Sesamum indicum	M	36	27.8	0.50±1.03
	F	32	37.5	0.84±1.44
	M+F	68	32.4 (92.4)	0.67±1.24
Ganoderma lucidum 25% in the diet	M	35	2.9	0.03±0.17
	F	38	0	0
	M+F	73	1.4	0.02±0.09
BP+Ganoderma lucidum	M	40	7.5	0.15±0.58
	F	40	5.0	0.13±0.56
	M+F	80	6.2 (17.8)[1]	0.14±0.57[2]

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection
Significantly different from the BP alone group at [1]P<0.05 and [2]P<0.005

are not a tool to examine cancer directly, were useful enzymes for indentifying carcinogenic or anticarcinogenic compounds *in vivo* (16, 17). The lung tumor model using mice has been used in carcinogenic or anticarcinogenic assays because lung tumors are induced in a short period (24, 25) and lung tumor incidence differs according to the age and strains of mouse, and the kind of carcinogen (26-29). It was also reported that BP had a long latent period for the development of lung tumors but it is an environmental carcinogen with which is humans easily come into contact (30). The authors previously reported the correlation between natural killer (NK) cell activity and lung adenoma incidence. In that experiment, NK cell activity became remarkably decreased from 4 weeks of age in a group of mice to which 1 mg urethane was administered within 24 hours after birth. The incidence of lung adenoma was 100 percent at week 9 in the urethane treated group. Considering that development of lung adenoma required a long-term latent period in the group treated with benzo(a)pyrene and also that NK cell activity normally reached a peak at week 6 and began descending at week 12 (21-23), we examined the lung tumor incidence in A/J, C57BL/6J, C57BR/cdJ and N:GP(S) at the 9th week after 0.5 mg or 1 mg BP injection. Because lung tumors were induced in A/J and N:GP(S) mice but in C57BL/6J and C57BR/cdJ they were not, to find the dose of BP inducing 50% tumor incidence, the dose response effect of lung tumor induction was tested in A/J and N:GP(S) mice. Forty micrograms of BP, which is the lowest dose in this experiment, induced a relatively high incidence which was above 50% in A/J mice. Five hundred micrograms of BP induced lung tumors and the incidence was about 50%. Therefore, we established a new medium-term lung tumor model in N:GP(S) newborn mice which were injected with 500 μg of BP after 9 weeks for the screening of carcinogenic or anticarcinogenic compounds (it was termed "Yun's anticarcinogenicity model") (31, 32).

To confirm the validity of this model, we tested various natural products some of which has shown anticarcinogenic effects in other long-term experiments. Ascorbic acid, which

had already shown anticarcinogenic activity in other model systems (33, 34) significantly inhibited lung tumor incidence. Red ginseng extract, which was previously reported to inhibit lung tumor incidence and increase the NK cell activity in a long-term anticarcinogenicity system (21-23), also induced the inhibition of lung tumor significantly. Fresh ginseng (4 years old) at 12.9 mg per ml of drinking water which is the equivalent dose to 1 mg of red ginseng extract did not show any inhibition. From these results, anticarcinogenic compounds of ginseng might be present or be present in greater amount in red ginseng (31, 32) and further investigation will be necessary to identify the reason why the lung tumor incidence in mice and odds ratios for human cancers were not decreased by fresh ginseng (35, 36).

Fresh carrot was administered to mice *ad libitum* at 4.4 g per mice daily, which dose was 8 times as much as 0.55 g/20g of body weight (37) being equivalent to a daily allowance of 6.6 μg of vitamin per 20 g of body weight suggested by NIH open formula No. 7 (38). In our experiment, carrot did not decrease lung tumor incidence. Beta-carotene was administered with the diet at 0.5 mg per 1 g of diet, being 63 times as much as a daily dietary allowance for mice, and did not induce any decrease of lung tumor incidence either. Vitamin A has been well known for its cancer preventive effect in oral, larynx, esophagus, stomach, bladder, breast, cervix, lung and colorectal cancers (39-44). Carotinoids are broken down in the small intestine, becoming reinaldehyde and then being converted into retinol. Beta-carotene was reported to be more important than retinol and retinol ester in preventing cancer because it is the most important caretonoid among diets, showing a lot of epidemiological evidence even thought it has none of the direct functions of vitamin A (45). It has been reported that beta-carotene inhibits ultraviolet (UV) induced cancer (46), but there have so far been no reports on the cancer preventing effect in animal systems except for UV-induced skin carcinogenesis. These results were paralleled with our present results, in which no decrease of lung tumor incidence was observed despite administration of beta-carotene at 60 times as much as the dietary allowance which was able not only to block the benzo(a)pyrene induced photocarcinogenesis but also to inhibit carcinogenesis in dark conditions.

The known functions of soybean lecithin are resolving cholesterol, promoting the cerebral functions, preventing senility, and working in close relation with vitamin E. Soybean lecithin contains 65% to 75% of phospholipids mainly composed of linoleic acid, and linoleic acid has been reported to decrease the incidence of papillomas in mice (47). In this system, soybean lecithin reduced the incidence of lung tumors significantly.

Sesamum indicum did not reduce the incidence of lung tumors in our results, but sesamol, the main ingredient of *Sesamum indicum*, has been reported to inhibit hepatocarcinogenesis using glutathione S-transferase placental form positive foci (48).

The polysaccharide fraction of *Ganoderma lucidum* was

Table VII. *The lung tumor incidence and multiplicity in N:GP(S) mice at the 9th week after benzo(a)pyrene injection combined with caffeine.*

Groups and treatment	Number of mice		Incidence (%)	Multiplicity (Mean±SD)
1% Gelatin	M	40	0	0
	F	40	0	0
	M+F	80	0	0
Benzo(a)pyrene	M	40	47.5	1.58±2.57
0.5 mg*	F	40	35.0	0.80±1.59
	M+F	80	41.3 (100)	1.19±2.08
Caffeine	M	30	0	0
1 mg/day	F	30	0	0
	M+F	60	0	0
2 mg/day	M	29	0	0
	F	30	0	0
	M+F	59	0	0
BP+caffeine				
1 mg/day	M	40	17.5	0.33±0.92
	F	40	20.5	0.40±1.08
	M+F	80	18.8 (46.0)[1]	0.37±1.00[1]
2 mg /day	M	39	5.1	1.10±1.39
	F	38	5.1	1.33±1.71
	M+F	78	5.1 (12)[1]	1.22±1.55[2]
13-Cis retinoic acid	M	20	0	0
240 mg/kg diet	F	20	0	0
	M+F	40	0	0
BP+13-cis retinoic acid				
	M	24	41.7	0.58±0.80
	F	20	55.0	1.65±2.00
	M+F	44	48.4 (117)	1.07±1.58

M: Male mice, F: Female mice
* mg/mouse, subcutaneous injection
Significantly different from the BP alone group at [1]P<0.05 and [2]P<0.005

innocuous, showing antitumor activity in transplanted tumor (49) by immunostimulation (50). Administration of *Ganoderma lucidum* powders showed inhibition of lung tumor in the present experiment. According to a report (51), spinach was a inducer of benzo(a)pyrene hydrolase activity and had a protective system against carcinogenesis. Another report (52) revealed that it contained antioxidant. However, no cancer preventive effect was proved in this experiment.

Caffeine has been reported to show an anticarcinogenic effect in mouse skin and lung cancer (53, 54) and also induced the same result in our experiment.

13-Cis retinoic acid has been reported to shows anticarcinogenic activity in N-butyl-N-(4-hydroxybutyl)-nitrosamine or 1-methyl-1-nitrosourea induced urinary bladder carcinogenesis, and skin carcinogenesis promoted by phorbol ester (55, 56). However, it did not show any inhibition of lung tumors in our experiment system. The difference in results is assumed to be

due to the possibility that the anticarcinogenicity of retinoids may differ according to the target organ, e.g. 13-cis retinoic acid did not induce the cancer preventive effect on mammary tumor but trimethylmethoxyphenyl derivatives of ethyl retinoid did (57).

The present study was carried out to establish a new medium term *in vivo* carcinogenic or anticarcinogenic model in newborn mice induced by benzo(a)pyrene and to confirm this model using various natural products some of which are known to be anticarcinogens. From these results, it is thought that this model will be useful in the detection of carcinogenicity or anticarcinogenicity of unknown chemicals.

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Inhibition by ginsenoside Rg_3 of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats

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The effects of concomitant use of bombesin and ginsenoside Rg_3 on the incidence of peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane were investigated in male inbred Wistar rats. From the start of the experiment, rats were given weekly s.c. injections of azoxymethane (7.4 mg/kg body weight) for 10 weeks and s.c. injection of bombesin (40 μg/kg body weight) every other day, and from week 20, s.c. injections of ginsenoside Rg_3 (2.5 or 5.0 mg/kg body weight) every other day until the end of the experiment in week 45. Bombesin significantly increased the incidence of intestinal tumors and cancer metastasis to the peritoneum in week 45. It also significantly increased the labeling index of intestinal cancers. Although administration of a higher dose of ginsenoside Rg_3 with bombesin had little or no effect on the enhancement of intestinal carcinogenesis by bombesin, the location, histologic type, depth of involvement, infiltrating growth pattern, labeling and apoptotic indices and tumor vascularity of intestinal cancers, it significantly decreased the incidence of cancer metastasis. These findings indicate that ginsenoside Rg_3 inhibits cancer metastasis through activities that do not affect the growth or vascularity of intestinal cancers.

Keywords: azoxymethane, bombesin, cancer metastasis, ginsenoside Rg_3, intestinal cancer

Introduction

Chemically induced carcinomas seldom metastasize to distant organs, lymph nodes, or the peritoneum. Usually, metastatic models are created in animals by transplantation of cancer cells. However, these models are quite different from the modes of metastasis in humans. Therefore, to investigate the mechanism of cancer metastasis and to develop antimetastatic agents, new metastatic models in animals are necessary. Recently, we developed a new metastatic model in rats [1]. We gave rats 10 weekly s.c. injections of azoxymethane (AOM) and s.c. injection of bombesin, a 14-amino-acid peptide, originally isolated from the skin of the frog *Bombina bombina*, every other day until the end of the experiment. Administration of bombesin significantly increases the incidence of intestinal cancers. Although it has no effect on the histologic features or depth of involvement of colon adenocarcinomas, it significantly increases the incidence of metastasis to the peritoneum. This bombesin-induced peritoneal metastasis is very similar to metastasis in

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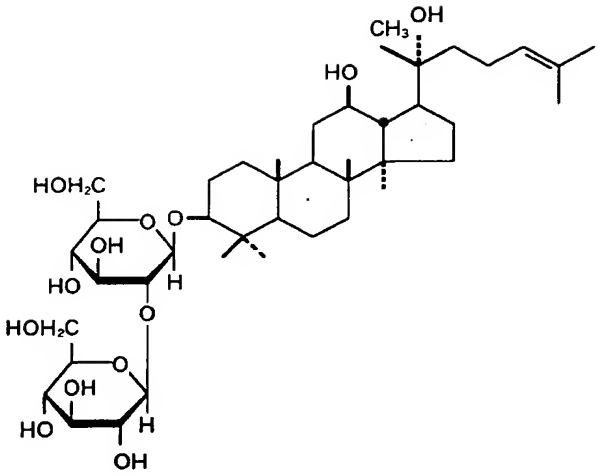

Figure 1. Structural formula of ginsenoside Rg_3.

humans. Therefore, this model is useful for the investigation of the mechanism of metastasis [2, 3].

A decoction of the roots of *Panax ginseng* C.A. MEYER (ginseng root) is a traditional Asian medicine with analeptic, stomachic, and erythropoietic properties [4]. Extensive analyses of the extract of ginseng root have indicated that ginsenosides mediate the biologic activities of ginseng root [5–8]. The ginsenosides are plant glycosides with an aglycone (protopanaxadiol or protopanaxatriol) possessing a dammarane skeleton. Recently, we found that ginsenoside Rg_3, a ginsenoside from *P. ginseng,* inhibits invasion by certain tumor cells without impairing cell growth *in vitro* [4, 9, 10]. Therefore, in the present study, we used a bombesin-induced cancer metastasis model to examine the effects of ginsenoside Rg_3 on development of intestinal cancers induced by AOM and their metastasis to the peritoneum in Wistar rats.

Materials and methods

Preparation of ginsenoside Rg_3

Ginsenosides were isolated from unprocessed and processed roots (white ginseng and red ginseng) of *P. ginseng* C.M. MEYER. Figure 1 shows the structural formula of ginsenoside Rg_3.

Animals

One hundred and twenty 6-week-old male inbred Wistar rats purchased from Japan SLC (Shizuoka, Japan) were used for this experiment. The animals were housed in suspended, wire-bottomed metal cages in our animal quarters at controlled temperature (20–22°C) and humidity (30–50%), with a 12 h–12 h light–dark cycle. Regular chow pellets (Nihon-Nosan, Yokohama, Japan) and normal tap water were supplied *ad libitum.*

Carcinogen and treatments

The animals were randomly divided into six groups of 20 rats each and given weekly s.c. injections of 7.4 mg/kg body weight of AOM (Sigma Chemical Co., Inc., St. Louis, MO) in 0.9% NaCl solution for 10 weeks, and received the following treatments for the duration of the experiment until the end of the experiment in week 45. Group 1, the control group, received only injections of olive oil. Group 2 received injections of bombesin alone. Groups 3 and 4 received injections of bombesin and 5.0 and 2.5 mg/kg body weight, respectively, of ginsenoside Rg_3. Groups 5 and 6 received injections of 5.0 and 2.5 mg/kg body weight, respectively, of ginsenoside Rg_3 without bombesin.

Bombesin (Sigma) at 40 μg/kg body weight and ginsenoside Rg_3 at 5.0 and 2.5 mg/kg body weight were prepared as suspensions in olive oil. Injections were given s.c. in a volume of 1 ml/kg body weight between 2 and 3 p.m. every other day at various sites. Bombesin and ginsenoside Rg_3 were injected from the start of the experiment and from week 20, respectively.

Histologic observations

The first intestinal tumor was found in a rat of group 2 killed in week 32, so rats that survived for more than 32 weeks were included in the effective numbers. Rats were killed when they became moribund, and surviving animals were killed at the end of week 45. The internal organs of all animals killed during the experiment in week 45 were carefully examined. The large and small intestines were opened, pinned flat on cork mat, and fixed with buffered picric acid–formaldehyde solution. Tumor-bearing areas and areas suspected of bearing lesions were excised and embedded in paraffin. Semiserial, 5-μm-thick sections were cut to expose the central part of the tumor or the stalk, when present, and were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from each segment of the fixed intestine with no visible tumors was cut into 3-mm-wide strips, which were embedded in paraffin. Thin sections were prepared and examined microscopically for tumor foci. All sections were examined without knowledge of their groups of origin.

Classification of intestinal tumors
Adenomas were defined histologically as lesions in which neoplastic cells were confined to the mucosal layer, while adenocarcinomas were defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to invade the submucosa or deeper layers. As reported previously [2], adenocarcinomas were further classified as either well-differentiated or mucinous carcinomas. In the former, tumor cells were found in acinar clusters simulating the glandular structures of normal intestinal mucosa. In the latter, mucin secretion was active, resulting in mucinous nodules containing large amounts of extracellular mucin with only a few isolated groups of tumor cells.

Grades of peritoneal metastasis
Grades of metastasis of intestinal adenocarcinomas to the peritoneum were classified according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus in Japan [11], as follows: P_1, metastatic nodules detectable only over the peritoneum near the primary cancer; P_2, a few metastatic nodules also detectable over the peritoneum far from the primary cancer; and P_3, many metastatic nodules also detectable over the peritoneum far from the primary cancer.

Patterns of infiltrating growth of adenocarcinomas
The predominant patterns of infiltrating growth into the surrounding tissue were also classified according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus in Japan [11], as follows: alpha, tumor shows expanding growth and a distinct border from the surrounding tissue; beta, this category is between alpha and gamma types; and gamma, tumor shows infiltrating growth and an indistinct border from the surrounding tissue.

Measurement of labeling index
The labeling indices of large and small intestinal adenocarcinomas were measured in week 45 with an immunohistochemical analysis kit for bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson, Mountain View, CA) [12, 13]. For the assay, 10 rats in each group were given only tap water for 12 h. Then rats received 1 ml/kg body weight of olive oil (group 1), 40 μg/kg body weight of bombesin (group 2), 40 μg/kg body weight of bombesin plus 5.0 mg/kg body weight of ginsenoside Rg_3 (group 3), 40 μg/kg body weight of bombesin plus 2.5 mg/kg body weight of ginsenoside Rg_3 (group 4), 5.0 mg/kg body weight of ginsenoside Rg_3 (group 5), or 2.5 mg/kg body weight of ginsenoside Rg_3 (group 6). One hour later, the animals received an i.p. injection of BrdU (20 mg/kg body weight) and were killed with ether 1 h later. The small and large intestines were removed and fixed in 70% ethanol for 4 h. Tumor-bearing small and large intestines were then embedded in paraffin. Thin sections of 3-μm thickness were immersed in 2 N HCl solution for 30 min and then in 0.1 M $Na_2B_4O_7$. Slides were also immersed in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and then treated with 10% horse serum. Specimens were incubated with anti-BrdU monoclonal antibody (diluted 1:20) for 2 h, washed, stained with biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA; diluted 1:200) for 30 min, and treated with avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min. The reaction product was detected with 3,3′-diaminobenzidine tetrahydrochloride. Cells that contained BrdU were identified by the presence of a dark pigment over their nuclei.

To analyse the labeling index of intestinal adenocarcinomas, we counted the number of BrdU-labeled and -unlabeled cells among 500 cancer cells. On the basis of these measurements, we calculated the labeling index as the percentage of BrdU-labeled cells per total number of cancer cells.

Measurement of apoptotic index
The apoptotic index of the adenocarcinomas were examined at week 45. The apoptotic index was measured with an *in situ* apoptotic detection kit (Oncor, Gaithersburg, MD) for assaying apoptotic cells by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA in 6-μm sections of fixed tissue obtained from the colon of 10 rats in each group [14]. Digoxigenin-labeled cells among 500 cells in the adenocarcinomas were counted. The apoptotic index was expressed as the percentage of labeled cells among the cells examined.

Measurement of tumor vascularity
Tumor vascularity of the intestinal adenocarcinomas was examined at week 45 [15]. Staining for vascular endothelial cells was performed on paraffin-embedded tissue sections using the avidin–biotin–immunoperoxidase complex technique. Six-micrometer thick sections, mounted on glass slides, were deparaffinized with xylene. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 5 min. After washing with phosphate-buffered saline, they were incubated in 10% normal bovine serum for 5 min, followed by incubation

overnight with anti-von Willebrand factor monoclonal antibody (F8/86; DAKO A/S, Denmark) at 1:25 dilution. Biotinylated goat anti-mouse immunoglobulin and anti-rabbit immunoglobulin (Dako LSAB kit; Dako Japan, Kyoto, Japan) were used as secondary antibodies. Peroxidase-conjugated avidin (Dako Japan) was used at a dilution of 1:500. Finally, 0.02% diaminobenzidine and 1% hydrogen peroxide (Dako Japan) in phosphate-buffered saline were used as the substrate. Normal mouse immunoglobulin G diluted to an equivalent protein concentration was used as a control in place of the primary antibody. The sections were counterstained with hematoxylin.

For assessment of tumor vascular density, slides were examined under low power (×40 to ×100) to identify the region of highest vessel density. The five most vascular areas within the adenocarcinomas for each slide were selected. A × 200 field in each of these five areas was counted, and the average counts were recorded. A vessel lumen was not required for identification of a microvessel; single cells or cell cluster were counted. Large vessels with thick muscular walls or with lumen greater than 50 μm were excluded from the count.

Statistical analysis

Data were analysed with the chi-square test, Fisher's exact probability test [16], or one-way analysis of variance with Dunn's multiple comparison [17]. Data are shown as means ± SE. Differences were considered to be significant at a calculated P value of < 0.05.

Results

Incidence, location, and histologic type of intestinal tumors

One rat in each of groups 1 and 5 and two rats in group 6 died before week 26. No tumors were found

Table 1. Body weight and incidence of intestinal tumors in AOM-treated rats

Group no.	Treatment[a]	Body weight (g)		Effective no. of rats	No. of rats with intestinal tumors (%)
		Initial	Week 45		
1	Olive oil	116 ± 3	328 ± 13	19	11 (58)
2	Bombesin	119 ± 2	340 ± 7	20	20 (100)[b]
3	Bombesin + Rg_3 (5.0 mg/kg)	120 ± 1	322 ± 3	20	20 (100)[b]
4	Bombesin + Rg_3 (2.5 mg/kg)	116 ± 2	320 ± 13	20	20 (100)[b]
5	Rg_3 (5.0 mg/kg)	116 ± 2	329 ± 18	19	13 (68)
6	Rg_3 (2.5 mg/kg)	114 ± 3	321 ± 15	18	12 (66)

[a]Treatment: groups 1 and 2, rats were given one s.c. injection of 7.4 mg/kg body weight of AOM a week for 10 weeks and also s.c. injections of 1 ml/kg body weight of olive oil (group 1) or 40 μg/kg body weight of bombesin (group 2) every other day until the end of the experiment; groups 3 and 4, rats were given one s.c. injection of 7.4 mg/kg body weight of AOM a week for 10 weeks and also s.c. injections of 40 μg/kg body weight of bombesin plus 5.0 mg/kg (group 3) or 2.5 mg/kg (group 4) body weight of Rg_3 every other day until the end of the experiment; groups 5 and 6, rats were given one s.c. injection of 7.4 mg/kg body weight of AOM a week for 10 weeks and also s.c. injections of 5.0 mg/kg (group 5) or 2.5 mg/kg (group 6) body weight of Rg_3 every other day until the end of the experiment.

[b]Significantly different from the value for group 1 at $P < 0.01$.

Table 2. Locations and histologic types of intestinal tumors in AOM-treated rats

Group no.	Treatment[a]	No. of intestinal tumors	Location (%)		Histologic type (%)	
			Small intestine	Large intestine	Adenoma	Adenocarcinoma
1	Olive oil	24	8 (33)	16 (67)	10 (42)	14 (58)
2	Bombesin	39	6 (15)	33 (85)	19 (49)	20 (51)
3	Bombesin + Rg_3 (5.0 mg/kg)	40	4 (10)	36 (90)	18 (45)	22 (55)
4	Bombesin + Rg_3 (2.5 mg/kg)	38	6 (16)	32 (84)	14 (37)	24 (63)
5	Rg_3 (5.0 mg/kg)	24	3 (12)	21 (88)	10 (42)	14 (58)
6	Rg_3 (2.5 mg/kg)	20	3 (15)	17 (85)	5 (25)	15 (75)

[a]For explanation of treatments, see Table 1.

Table 3. Incidence and grades of peritoneal metastasis of intestinal adenocarcinomas in AOM-treated rats

Group no.	Treatment[a]	No. of rats with adenocarcinoma	No. of rats with peritoneal metastasis (%)	Grade of metastasis[b] (%)		
				P_1	P_2	P_3
1	Olive oil	10	1 (10)	1 (100)	0 (0)	0 (0)
2	Bombesin	14	8 (57)[c]	0 (0)	0 (0)	8 (100)
3	Bombesin + Rg_3 (5.0 mg/kg)	16	2 (13)[d]	1 (50)	0 (0)	1 (50)
4	Bombesin + Rg_3 (2.5 mg/kg)	18	7 (39)	0 (0)	0 (0)	7 (100)
5	Rg_3 (5.0 mg/kg)	12	2 (16)	1 (50)	0 (0)	1 (50)
6	Rg_3 (2.5 mg/kg)	10	1 (10)	0 (0)	0 (0)	1 (100)

[a]For explanation of treatments, see Table 1.
[b]Grade of metastasis: P_1, metastatic nodules detectable over the peritoneum near the primary cancer; P_2, a few metastatic nodules detectable over the peritoneum far from the primary cancer; P_3, many metastatic nodules detectable over the peritoneum far from the primary cancer.
[c]Significantly different from the value for group 1 at $P < 0.05$.
[d]Significantly different from the value for group 2 at $P < 0.05$.

in these rats, which were excluded from the effective numbers. In week 45, administration of bombesin or ginsenoside Rg_3, or both, had little or no effect on the body weight of rats (Table 1).

In group 1 (olive oil), intestinal tumors were found in 11 (58%) of 19 rats examined. In group 2 (bombesin alone), the incidence of intestinal tumors was significantly higher than in group 1. Combined use of bombesin and ginsenoside Rg_3 (groups 3 and 4) had no significant effect on the incidence of intestinal tumors compared with that in group 2. The incidence in rats treated with ginsenoside Rg_3 alone (groups 5 and 6) did not differ significantly from that of rats in group 1.

There were no significant differences in the location of intestinal tumors or the distributions of adenomas and adenocarcinomas among the six groups (Table 2).

Incidence and grade of peritoneal metastasis

In group 1 (olive oil alone), peritoneal metastasis of intestinal cancers was found in only 1 (10%) of 10 rats bearing intestinal adenocarcinomas (Table 3). In group 2 (bombesin alone), the incidence of peritoneal metastasis was significantly higher than in group 1: peritoneal metastases were found in 8 (57%) of 14 cancer-bearing rats. Concomitant use of bombesin and ginsenoside Rg_3 at 5.0 mg/kg body weight (group 3), but not at 2.5 mg/kg body weight (group 4), resulted in a significantly lower incidence of peritoneal metastasis compared with that in group 2. Treatment with ginsenoside Rg_3 at both dosages (groups 5 and 6) had no significant effect on the incidence of peritoneal metastasis compared with that in group 1.

In group 2 (bombesin alone), many metastatic nodules were found far from the primary adenocarcinomas in all eight rats with peritoneal metastases. However, in group 3 (bombesin plus ginsenoside Rg_3 at 5.0 mg/kg body weight), a few metastatic nodules were detected far from or near the primary cancers. No metastases to the liver or lung were found macroscopically or microscopically.

Effects on histologic features, labeling index, apoptotic index and tumor vascularity of cancers

There were no differences in the location, histologic type, the depth of involvement, infiltrating growth pattern and venous invasion of the intestinal adenocarcinomas among six groups (Table 4). Although treatment with bombesin alone (group 2) had no significant effect on the histologic features of intestinal cancers, it significantly increased their labeling indices (Table 5). Administration of bombesin and ginsenoside Rg_3 at both dosages (groups 3 and 4) had no significant effect on either the histologic features or labeling indices of intestinal adenocarcinomas compared with those in group 2. Administration of bombesin or ginsenoside Rg_3 had no significant effects on the apoptotic index and tumor vascularity of adenocarcinomas.

Discussion

Our present study shows that bombesin enhances the development and peritoneal metastasis of intestinal adenocarcinomas and that ginsenoside Rg_3 attenuates the enhancement of peritoneal metastasis induced by bombesin, although it has little or no effect on the incidence, histologic type, the depth of

Table 4. Effects of bombesin and Rg_3 on the locations, histologic types, depths of involvement, infiltrating growth patterns and venous invasions of intestinal adenocarcinomas

Group no.	Treatment[a]	No. of adenocarcinomas	Location (%)		Histology (%)		Depth of involvement (%)		Infiltrating growth[b] (%)			Venous invasion	
			Small intestine	Large intestine	Well differentiated	Mucinous	Submucosa or muscle layer	Subserosa or deeper	alpha	beta	gamma	(+)	(−)
1	Olive oil	14	8 (57)	6 (43)	8 (57)	6 (43)	8 (57)	6 (43)	4 (29)	6 (42)	4 (29)	0 (0)	14 (100)
2	Bombesin	20	6 (30)	14 (70)	11 (55)	9 (45)	13 (65)	7 (35)	5 (25)	8 (40)	7 (35)	0 (0)	20 (100)
3	Bombesin + Rg_3 (5.0 mg/kg)	22	4 (18)	18 (82)	12 (55)	10 (45)	13 (59)	9 (41)	5 (23)	9 (41)	8 (36)	0 (0)	22 (100)
4	Bombesin + Rg_3 (2.5 mg/kg)	24	6 (25)	18 (75)	9 (38)	15 (62)	13 (54)	11 (46)	6 (24)	9 (38)	9 (38)	0 (0)	24 (100)
5	Rg_3 (5.0 mg/kg)	14	3 (21)	11 (79)	5 (36)	9 (64)	7 (50)	7 (50)	3 (21)	7 (50)	4 (29)	0 (0)	14 (100)
6	Rg_3 (2.5 mg/kg)	15	3 (20)	12 (80)	9 (60)	6 (40)	9 (60)	6 (40)	4 (27)	7 (46)	4 (27)	0 (0)	15 (100)

[a]For explanation of treatments, see Table 1.

[b]Pattern of infiltrating growth: alpha, tumor shows expanding growth and a distinct border from the surrounding tissue; beta, this category is between the alpha and gamma types; gamma, tumor shows infiltrating growth and an indistinct border from the surrounding tissue.

Table 5. Effects of bombesin and Rg_3 on labeling index, apoptotic index and tumor vascularity of intestinal carcinomas

Group no.	Treatment[a]	Labeling index, %	Apoptotic index, %	Vessel counts, no./field
1	Olive oil	30.6 ± 1.6 (7)[b]	4.6 ± 0.5 (7)	47.3 ± 3.6 (7)
2	Bombesin	43.2 ± 2.0[c] (10)	4.4 ± 0.6 (10)	53.4 ± 7.3 (10)
3	Bombesin + Rg_3 (5.0 mg/kg)	45.0 ± 2.0[d] (9)	5.0 ± 0.8 (13)	46.0 ± 5.8 (13)
4	Bombesin + Rg_3 (2.5 mg/kg)	46.0 ± 2.7[d] (11)	4.2 ± 0.6 (13)	44.7 ± 7.0 (13)
5	Rg_3 (5.0 mg/kg)	27.8 ± 1.4 (7)	4.9 ± 0.5 (7)	44.0 ± 6.5 (7)
6	Rg_3 (2.5 mg/kg)	17.8 ± 1.9 (6)	4.7 ± 0.8 (9)	46.9 ± 7.7 (9)

[a]For explanation of treatments, see Table 1.
[b]Numbers in parentheses are numbers of adenocarcinomas examined.
[c,d]Significantly different from the value for group 1: [c]$P < 0.01$, [d]$P < 0.001$.

involvement, infiltrative growth pattern, grade of venous invasion, labeling index, apoptotic index and tumor vascularity of intestinal cancers. These results suggest that ginsenoside has an antimetastatic effect.

Although ginsenoside Rg_3 is water-insoluble, Shinkai *et al.* [4] found that this compound is dissolved in dimethyl sulfoxide. In the present work, we administered ginsenoside Rg_3 as suspension in olive oil and found that this compound has an antimetastatic effect. However, there were no reports on the concentration of this compound at the tumor periphery and in the peritoneal cavity after s.c. injection. Further investigation is needed.

Although the exact mechanisms by which ginsenoside Rg_3 attenuates peritoneal metastasis of intestinal adenocarcinomas are unclear, at least three possible explanations may be considered. One possibility is the effect of ginsenoside on enhancement of nitric oxide production. Kim *et al.* [18] reported that ginsenoside releases endothelium-derived nitric oxide from the aorta. Fan *et al.* [19] found that ginsenoside enhances the production of nitric oxide from interferon-gamma-activated macrophages or a macrophage cell line, RAW264–7, and that it also enhances the production of nitric oxide from macrophages co-cultured with nonadherent spleen cells. Production of endogenous nitric oxide was shown to reduce metastatic potential in tumor cells. This effect was mediated through inhibition of platelet aggregation [20, 21] or induction of apoptosis [22, 23]. However, there have been no reports on the effects of ginsenoside Rg_3 on nitric oxide production.

A second possible explanation of how ginsenoside attenuates bombesin-enhanced peritoneal metastasis is its effect on the adhesion of tumor cells or on tumor-induced angiogenesis. Mochizuki *et al.* [24] examined the effect of ginsenoside Rg_3 on lung metastasis by two highly metastatic tumor cells, B16-BL6 melanoma and colon 26-M3.1 carcinoma, in syngeneic mice. Ginsenoside Rg_3 significantly inhibits the invasion of B16-BL6 cells into the reconstituted basement membrane in a dose-dependent manner. In an experimental metastasis model using B16-BL6 melanoma, intravenous or oral administration of ginsenoside Rg_3 induces a significant decrease in lung metastasis of B16-BL6 melanoma. In an *in vitro* analysis, ginsenoside Rg_3 significantly inhibits adhesion to fibronectin and laminin by B16-BL6 melanoma. Mice treated with the drug after tumor inoculation exhibit a significant decrease in the number of blood vessels oriented toward the tumor mass with no decrease in tumor size. These findings suggest that ginsenoside Rg_3 possesses an ability to inhibit the lung metastasis of tumor cells and that the mechanism of their antimetastatic effect is related to inhibition of the adhesion and invasion of tumor cells and to anti-angiogenic activity. In the present work, however, administration of ginsenoside Rg_3 had no significant effects on tumor-induced angiogenesis and adhesion between tumor cells (Tables 4 and 5).

The third possible explanation for inhibition of metastasis by ginsenoside Rg_3 is its effect on intracellular calcium ($[Ca^{2+}]_i$). Elevated $[Ca^{2+}]_i$ is thought to be closely related to tumor cell invasion. Imamura *et al.* [25] found that serum is required for the *in vitro* invasion of a highly invasive clone, MM1. Addition of serum to a MM1 cell suspension induces an increase in the intracellular pH (pH_i) as well as a transient elevation of $[Ca^{2+}]_i$, whereas addition of serum to a poorly invasive clone does not result in a pH_i change or $[Ca^{2+}]_i$ spike. Bombesin was shown to increase $[Ca^{2+}]_i$ [26]. In contrast, Nah *et al.* [27]

reported that a crude extract from ginseng root inhibits high-threshold, voltage-dependent Ca^{2+} channels through an unknown receptor linked to a pertussis toxin-sensitive G protein. Recently, we examined the effect of ginsenoside Rg_3 on $[Ca^{2+}]_i$ of rat ascites hepatoma cells (MM1) [4]. The addition of 1-oleoyl-lysophosphatidic acid to a cell suspension resulted in an almost instantaneous increase in $[Ca^{2+}]_i$. However, when MM1 cells were pretreated with ginsenoside Rg_3, the $[Ca^{2+}]_i$ spike induced by 1-oleoyl-lysophosphatidic acid was completely abolished. In the present study, it was impossible to cultivate the adenocarcinoma cells that developed in the intestine in Wistar rats treated with AOM. Therefore, we could not confirm the possibility of reduction of $[Ca^{2+}]_i$ level by ginsenoside Rg_3. But these results suggest that ginsenoside Rg_3 attenuates cancer cell invasion through suppression of the $[Ca^{2+}]_i$ spike.

In conclusion, our present work shows that administration of ginsenoside Rg_3 significantly decreases the incidence of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas, although it does not significantly affect bombesin-enhanced intestinal tumorigenesis. These findings indicate that ginsenoside Rg_3 has an antimetastatic action, which may be mediated through decreased intracellular calcium levels.

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